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1) HUBL et al., CYTOMETRY Vol 30 (2): 72-84 (April 15, 1997).

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3) FESTIN et al., Journal of Immunological Methods Vol 177 (1-2): 215-224 (December 28, 1994).

4) KEENEY et al., CYTOMETRY Vol 34 (2): 61-70 (April 15, 1998).

5) MACEY et al., Journal of Immunological Methods Vol 204 (2): 175-188 (May 26, 1997).

6) McCARTHY et al., Journal of Immunological Methods Vol 163 (2): 155-160 (August 9, 1993).

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The use of caprylic acid for the extraction of the immunoglobulin fraction from egg yolk of chickens immunised with ovine α-lactalbumin

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Abstract

The extraction and purification of serum-derived immunoglobulin fraction in the egg yolk of hens by the combined treatment of the raw egg yolk with caprylic (octanoic) acid and ammonium sulphate is described. This simple two-step method proved to be both rapid, reproducible and suitable for batch processing of pooled egg yolk. The method recovered in excess of 130 mg of immunoglobulin per egg yolk.

Two chickens were inoculated at two weekly intervals with 100 μg each of ovine α-lactalbumin over a ten week period. The α-lactalbumin antigen was purified by a hydrophobic-interaction chromatographic procedure and further purified by a gel excision-elution process.

No precipitating antibodies could be demonstrated in gel diffusion techniques with this antibody. The specificity and specific activity of the antibody were monitored by western blotting and demonstrated the presence of highly specific antibodies to ovine α-lactalbumin in the treated egg yolk. The extraction procedure had no adverse effects on antibody titre.

We concluded, and confirmed previous reports, that the use of chickens for the production of highly specific antibodies to mammalian proteins with particular reference to milk proteins presented numerous advantages over conventional procedures.

Keywords: Chicken egg yolk; Immunoglobulin extraction; Caprylic acid; α-Lactalbumin, ovine; Immunization

1. Introduction

Traditionally, analytical procedures for the estimation of levels of milk proteins, α-lactalbumin, β-lactoglobulin, lactoferrin, caseins as well as serum-derived proteins in milk of domesticated animals have largely depended upon electrophoretic methods (Ng-kwai-hang and Kroeker, 1984; Basch et al., 1985). Meisel (1990) has suggested that immunological analytical procedures have not found favour with the dairying industry

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simply because antisera to individual milk proteins are not available commercially. The use of antibodies to bovine lactoferrin derived from the eggs of immunised hens and their use in an enzyme-linked immunosorbent assay (ELISA) was described in the same report. Polson et al. (1980) likened the yolk of birds to colostrum in early post-partum milk. In the egg, serum-derived proteins, notably immunoglobulins, concentrate in the yolk thereby providing passive immunity to the developing chick. The levels of immunoglobulin in the egg yolk have been reported to be as high as, or even greater than, those in chicken serum (Otani et al., 1989). In an earlier report, Polson et al. (1980) listed a number of advantages of using chickens as a source of antibody. These included the rapid production of large volumes of highly concentrated egg yolk-containing immunoglobulin, the minimum of invasive procedures such as frequent inoculation and bleeding. Others subsequently have included the phylogenetic distance between mammals and birds as a two-fold advantage; low levels of antigen in the inoculum (Gassmann et al., 1990), and a high probability of high antigenicity of mammalian derived antigen in immunised hens (Vieira et al., 1984). The major disadvantage associated with antibody production in chickens, and perhaps the single reason for its lack of widespread use, is the difficulty in the extraction and purification of the immunoglobulin fraction from the yolk (Hassl and Aspock, 1988; Ntakarutimana et al., 1992). Moreover many of these methods are laborious and not applicable for large scale purification.

In this present report we describe the purification of ovine α-lactalbumin to raise specific antibodies in the egg yolk of chickens, and a simple rapid two-step procedure utilising caprylic acid for extracting and ammonium sulphate for further purifying these antibodies from harvested egg yolk. The aim of the study was to investigate the feasibility of employing hens in the production of specific antibodies to milk and other mammalian proteins for the subsequent development of immunochemical assays for use in milk composition and cell culture studies.

2. Materials and methods

2.1. Purification of ovine α-lactalbumin

Fresh sheep milk was centrifuged for 15 min at $1000 \times g$ and poured through four layers of cheese cloth to remove fat. The pH of the skim milk was adjusted to 4.3 with 1 N HCl and centrifuged for 20 min at $6000 \times g$. The supernatant was filtered through Whatman #1 filter paper and concentrated to approximately 60% of the original skim milk volume using an Amicon spiral ultrafiltration cartridge system, (Cartridge type S1Y10, Amicon, MA, USA), with a molecular weight cut off of 10 kilodaltons (kDa). The concentrated whey preparation was dialysed overnight against 6 vols. of Milli Q water, freeze dried and stored in a desiccator at 4°C until required. Ovine α-lactalbumin was purified from the whey preparation by a hydrophobic interaction chromatographic procedure (Lindahl and Vogel, 1984).

The freeze dried whey preparation was reconstituted in 250 mM Tris-350 mM EDTA buffer pH 7.5 prior to chromatography. Eluted fractions containing α-lactalbumin, determined by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE), were pooled, desalted and concentrated in an Omegacell ultrafiltration system with a molecular cutoff of 5 kDa (Filtron Technology, Clinton, MA, USA). Pooled fractions were stored in 1 ml ampoules at −20°C. Approximately 1.2 mg of the semipurifed α-lactalbumin in 1 ml of a mixture of sample and ×5 concentrated SDS buffer (4:1), was loaded on to a 160 × 125 mm 15% polyacrylamide gel slab (Laemmli, 1970) with a 145 mm wide preparative well formed in the stacking gel and run overnight at 50 V constant voltage on a Bio-Rad Protean II system (Bio-Rad Labs, Hercules, CA, USA.). At the completion of the separation, two 5 mm wide vertical slices were removed from each side of the gel slab.

The separated proteins in the gel slices were visualised by Coomassie blue staining (Neuhoff et al., 1988), and the α-lactalbumin band excised from the gel slab as previously described (McLaren et al., 1987). The excised acrylamide strip

was cut into 30 mm long segments and loaded into a 3 ml hypodermic syringe. The acrylamide segments were squeezed through the syringe nozzle into spin-X 0.22 μm cellulose acetate centrifuge filter units (Costar, Cambridge, MA, USA). A 4 mm diameter circle of glass filter paper was placed on the top of the cellulose acetate membrane at the base of each filter unit prior to loading the crushed acrylmamide. 200 μl of Milli Q water was added to the acrylamide in each unit and left at 4°C for 2 h. The filter units were centrifuged for 10 min at 6500 rpm in an MSE Micro Centaur centrifuge. The filtrate from each unit was pooled, mixed and dried down on a Savant SpeedVac rotory concentrator (Savant Instruments, Farmingdale, NY, USA). The purified α-lactalbumin was reconstituted in Milli-Q water.

2.2. Immunisation of chickens

The immunisation schedule was modified from the procedure described by Harboe and Ingild (1973) for generating specific polyclonal antibodies in the serum of rabbits. Two, prelaying, Ross brown pullets, approximately 20 weeks old, were inoculated at 2-weekly intervals for a total 10 weeks. Each bird was inoculated by multiple intramuscular site injections in the pectoral muscle with 100 μg of purified ovine α-lactalbumin in a mixture of equal amounts of Freund's complete and incomplete adjuvant. 1 ml of blood was removed from the brachial vein of each animal 10 days following the third and fifth inoculation. The serum was monitored for antibody activity by ELISA and Western blotting. Egg collection commenced immediately after the fourth inoculation.

2.3. Extraction and purification of egg yolk immunoglobulin

Eggs collected from immunised hens were processed in batches of 10. Each batch yielded a harvested egg yolk volume between 150 ml and 190 ml. The pooled egg yolk from each bird was diluted with 2 vols. of phosphate-buffered saline (PBS), pH 7.5 (Polson et al., 1980). The caprylic acid procedure for precipitating the egg yolk immunoglobulin fraction was modified from the method of Steinbuch and Audran (1969). The pH of the diluted egg yolk was adjusted to 4.6 with acetic acid and caprylic acid was slowly added dropwise, 0.6 ml/min, to give a final caprylic acid concentration of 6% (v/v). The mixture was stirred at room temperature for 2 h and centrifuged at 14,000 × g for 30 min. The resultant internatant, containing the immunoglobulin fraction, was collected through glass wool to remove the bright yellow oil layer on the surface. The pH of the extract was adjusted to 7.5 with 1 M Tris and centrifuged for 10 min at 14,000 × g. The preparation was cooled to 4°C and ammonium sulphate was added to a concentration of 1.75 M., allowed to stir at 4°C for 1 h and centrifuged at 14,000 × g for 20 min. The supernatant was discarded and the pellet washed by resuspending in 1.75 M ammonium sulphate and recentrifuged. The washed pellet was dissolved in PBS pH 7.5 to a volume equal to that of the undiluted harvested egg yolk, shell frozen and finally freeze dried.

The yolks from six eggs from one bird were collected over a single week and each egg processed individually to ascertain the reproducability of the extraction procedure. Subsamples were obtained from each step of the IgG extraction procedure for analysis.

2.4. Goat antichicken IgG antiserum

A 4-year-old non-lactating female Saanen goat was inoculated with chicken IgG (Sigma Chemical Co., St. Louis, MO, USA). The inoculation schedule was the same as that described for the chickens. The initial inoculum comprised of 1.5 ml of a homogenised mixture of 5 mg of chicken IgG in 500 μl of saline and 500 μl each of Freund's complete and incomplete adjuvant. In subsequent inoculations, the complete adjuvant was omitted from the inoculum. 10 ml of blood was obtained via the jugular 10 days after the third and fourth inoculation for estimation of antibody activity by simple immunodiffusion. Approximately 500 ml of blood was withdrawn from the jugular following the fifth inoculation.

2.5. Total protein estimation

Sample protein concentrations were determined by a modified Pierce bicinchoninic acid

(BCA) assay (Morton and Evans, 1992), and by the standard method according to the manufactures instructions (BCA protein assay reagent instruction manual # 23225x, Pierce, Rockford, IL, USA). In both assays the microtiter plate protocol was followed. The microtiter plates were incubated at 37°C for 2 h and sample absorbance values read at 595 nm on a Bio-Rad microtiter plate reader (Bio-Rad 3550, Bio-Rad Labs., Hercules, USA). The protein concentration of each sample was determined from a standard curve generated by a range of bovine serum albumin (BSA) concentrations from 0.25 mg to 2 mg/ml. The BSA standard was supplied with the BCA assay kit. Sample and BSA dilutions were prepared in 2% sodium dodecylsulphate (SDS) for the modified procedure and in phosphate buffered saline (PBS) for the conventional assay. Each diluent was used as a blank.

2.6. Polyacrylamide gel electrophoresis and electrophoretic transfer of proteins

Samples were electrophoresed on 85 × 50 mm polyacrylamide gel slabs (Bio-Rad Protean II mini gel system) after the method described by Laemmli (1970). Since the heavy chain of chicken IgG has a M_r similar to that of serum albumin, 67–70 kDa (Gassmann et al., 1990), samples were prepared under both mercaptoethanol reduced and non-reducing conditions. Gels were either stained with Coomassie blue (Neuhoff et al., 1988) or separated proteins in gels were electrophoretically transferred on to nitrocellulose membranes. Electrophoretic transfers were performed on a LKB Muliphore II Nova Blot system (LKB-Produkter, Bromma, Sweden), using a Tris-glycine continuous buffer system containing 20% methanol (Towbin et al., 1979). Sodium dodecylsulphate was omitted from the transfer buffer. Gels were electroblotted for 35 min at 0.8 mA per cm^2. The nitrocellulose membranes, NC-Extra 22 mm (Sartorius, Gottingen, Germany), were stained for 10 min in 0.1% amido black in 45% methanol containing 10% acetic acid (Schaffner and Weissman, 1973), or probed with chicken anti-ovine α-lactalbumin antibodies after the method of Ventling and Hurley (1988). The second antibody, horseradish peroxidase conjugated goat anti-chicken IgG (ICP, Auckland, New Zealand), was used at a concentration of 1:1000.

2.7. Immunoglobulin G assay

The total IgG concentration in samples was estimated by the Rocket immunoelectrophoresis assay of Laurell (1966). Goat anti-chicken IgG antisera was used in the assay at a concentration of 0.3% v/v. Each sample was diluted 1/20 with PBS and carbamylated prior to assay by adding 2 vols. of 2 M potassium cyanate to one volume of diluted sample and incubating for 1 h at 45°C (Weeke, 1968). A Tris-tricine buffer, pH 8.6, was used in the preparation of the agarose gel and also for the electrode buffer (Monthony et al., 1978). Rocket immunoelectrophoresis was performed in the Bio-phoresis Horizontal Electrophoresis Cell (Bio-Rad Laboratories, Hercules, CA, USA), at 1.5 V/cm for 5 h. Chicken IgG (Sigma Chemical Co., St. Louis, USA.) was used as standard in the range 200–800 μg/ml generating rocket heights from 10 to 50 mm. The area of each rocket was determined by triangulation, the base measured at 50% of the rocket height. The relationship of rocket area and antigen concentration of the IgG standards was used to generate a standard curve from which the IgG concentration in the samples was determined. Chicken sera, diluted egg yolk and serum from immunised goats were monitored for specific antibody activity by immunoelectrophoresis (Arquembourg et al., 1970), and by a simple qualitative Ouchterlony immunodiffusion procedure. In the immunodiffusion procedure, three agar gel buffer systems were employed; 0.15 M NaCl, 1.5 M NaCl (Polson et al., 1980) and 5% w/v polyethylene glycol 6000 (Ntakarutimana et al., 1992).

2.8. Estimation of levels of specific specific antibody activity by enzyme-immunoassay (ELISA)

A simple antibody capture assay (Harlow and Lane, 1988) was used to determine the level of specific antibody in yolk extracts and serum. Briefly, the purified antigen was coated to the

Table 1
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Table 2
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Total IgG (mg)	
IgG/protein (%	
Titre (dilution)	

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Table 1
Mean total protein and IgG concentrations from chicken serum and raw egg yolk

Sample	Mean protein concentration (mg/ml)	Mean IgG concentration (mg/ml)	Mean IgG concentration % of protein concentration	Mean egg yolk volume (ml)	mean total IgG/egg (mg)
Serum	41.9 ± 0.3	21.6 ± 0.7	52 ± 1		
Raw egg yolk	113 ± 2	19.3 ± 0.9	17.1 ± 0.8	19.3 ± 0.2	371 ± 20

The raw egg yolk values (mean ± SEM) were obtained from the yolks of six eggs from the same bird collected over 1 week. The serum samples ($n = 3$) were collected from one bird over a 5 week period. Samples were assayed for protein by the modified BCA of Morton and Evans (1992) and IgG levels determined by rocket immunoelectrophoresis (Laurell, 1966).

base of the microtitre plate well at 100 ng/well. Following overnight incubation at 4°C, the plates were blocked for 2 h at room temperature in 0.1% polyvinyl pyrrolidole 25 (PVP) to minimise non-specific binding. Samples of extract and serum were diluted to 1/200 and 1/500 respectively in diluent buffer, 0.1 M PBS, pH 7.5, containing 0.1% PVP. 100 μl of PBS diluent buffer was added to each well. 100 μl of the diluted sample was added to the first well of each well column, mixed and 100 μl serially transferred down the well column to generate a doubling dilution series. A second antibody, goat anti-chicken IgG horseradish peroxidase conjugate was used at a concentration of 1:1000 and incubated for 2 h. The substrate for the enzyme detection was 3, 3', 5, 5'-tetramethylbenzidine (TMB) and used according to the manufacturer's instructions (Bio-Rad laboratories, Hercules, CA, USA). The reaction was stopped with 1 N sulphuric acid and the plates were read at 450 nM with reference wavelegth 595 nM on a Bio-Rad 3550 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). A straight line equation was fitted to the data using the relationship log dilution as a function of absobance. The sample titre was taken as the x axis intercept of the computed line of best fit.

3. Results and discussion

Total protein and IgG concentrations in chicken serum and egg yolk are shown in Table 1. The mean IgG concentration was similar in both serum and egg yolk, 21.6 and 19.3 mg/ml, respectively. The serum IgG concentration was within the range reported by Kowalczyk et al. (1985), 5.3–43.3 mg/ml with a mean value of around 16.0 mg/ml. The chicken egg yolk IgG levels published in the literature also show considerable variation (Losch et al., 1986). Rosol et al. (1993) reported IgG concentrations in egg yolk of 20–25 mg/ml and Jensenius et al. (1981) reported values between 10 and 15 mg/ml after sodium sulphate precipitation. Total IgG per egg yolk appear to be in the region of 100–250 mg (Carroll and Strollar, 1983; Stuart et al., 1988; Gassmann

Table 2
Average total protein, IgG and antibody titre of six eggs from one bird

	REY	CAI	ASP
Total protein (mg)	1782 ± 18	313 ± 6	214 ± 4
Total IgG (mg)	371 ± 20	139 ± 5	136 ± 5
IgG/protein (%)	21 ± 1	45 ± 2	64 ± 2
Titre (dilution)	1/6339 ± 286	1/6182 ± 118	1/6218 ± 100

Egg yolks were processed individually by the caprylic acid-ammonium sulphate IgG extraction procedure. Total protein determined by the Pierce BCA assay, IgG by Rocket immunoelectrophoresis and titre from specific ELISA. Values are mean ± SEM ($n = 6$)
REY, raw egg yolk; CAI, caprylic acid internatant; ASP, reconstituted ammonium sulphate precipitate.

et al., 1990; Hatta et al., 1990; Lee et al., 1991; Yokoyama et al., 1993). In the present study the average content of IgG prior to extraction was estimated to be 371 ± 20 mg (mean ± SEM) per egg.

Treatment of six separate egg yolk preparations with caprylic acid reduced the mean total protein to 17.6 ± 0.4% of that of the original harvested egg yolk. This was further reduced after ammonium sulphate precipitation to 12 ± 0.3% and the mean ratio of IgG to total protein increased to more than three-fold (Table 2). The average yield of IgG from the six preparations was 136 ± 5 mg per egg, demonstrating the reproducability of the extraction procedure. The extraction process did not appear to effect antibody titre as assessed by specific ELISA (Table 2).

Extraction and purification of the immunoglobulin fraction from the egg yolks of immunised hens has been the focus of a number of reports (Ntakarutimana et al., 1992; Yokoyama et al., 1993; Akita and Nakai, 1993). The initial step in most egg yolk immunoglobulin extraction procedures involves the separation of proteins, principally albumin and lipoproteins, from IgG. The caprylic acid treatment used in the present study was adated from the method of Steinbuch and Audran (1969), optimised for the purification of IgG from rabbit serum (Borissenko, unpublished observations). It acted to precipitate the majority of both high and low molecular weight proteins in the raw egg yolk. The IgG fraction, along with two major bands, were retained in the internatant (Fig. 1). The intensities of both these bands appeared to be further reduced but not completely removed in the final preparation by ammonium sulphate precipitation.

Single immunoprecipitin arcs were obtained with the goat antiserum reacting with chicken serum, raw egg yolk and purified chicken IgG (Fig. 2), confirming homology between egg yolk-derived immunoglobulin and serum IgG as previously published (Hatta et al., 1990). We were unable to demonstrate precipitating antibodies to ovine α-lactalbumin in the immune egg yolk preparations. The inability of chickens to produce visible immune precipitation complexes in gel diffusion procedures has been previously documented (Ntakarutimana et al., 1992). Precipitating antibodies were generated in the egg yolk of chickens inoculated with viral antigens (Polson et al., 1980). However, it has been suggested that antigens with molecular weights of less than 30 kDa do not evoke precipitating antibodies and that the general immune response in chickens is

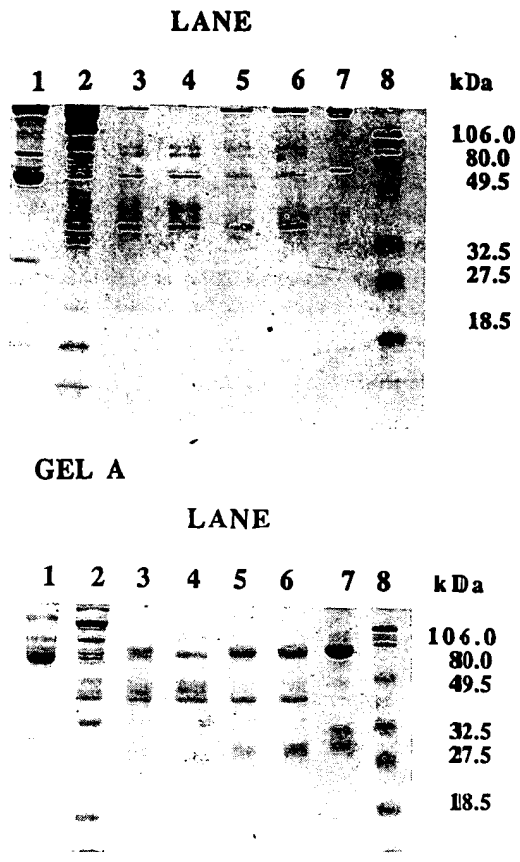

Fig. 1. SDS-PAGE of samples from egg yolk immunoglobulin purification. Each lane was loaded with 10 μg of protein. The acrylamide monomer concentration of each gel was 12.5%. Gel B contained mercaptoethanol treated samples. Lane 1, chicken serum; lane 2, untreated egg yolk; lane 3, caprylic acid internatant; lane 4, ammonium sulphate supernatant; lane 5, ammonium sulphate pellet redissolved in PBS; lane 6, as 5 following lyophilisation; lane 7, purified chicken serum IgG (Sigma); lane 8, prestained molecular weight markers (Bio-Rad).

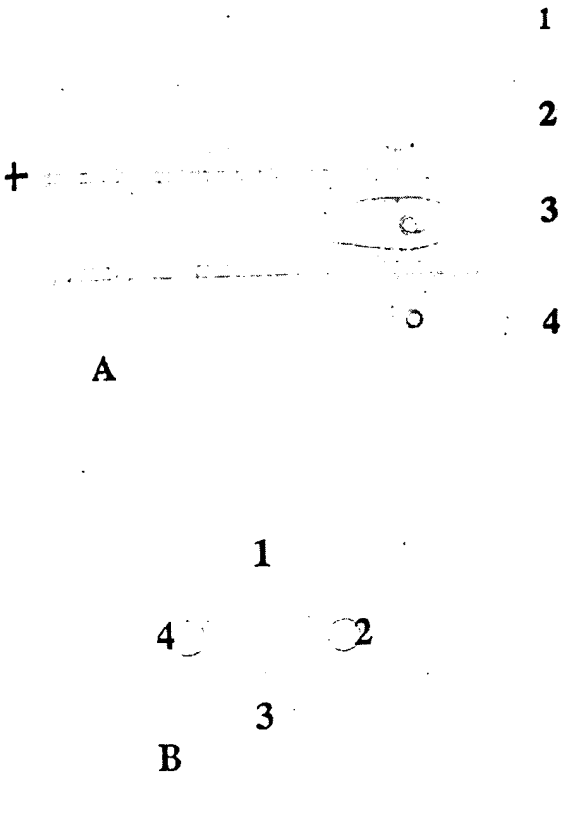

Fig. 2. *A*: immunoelectrophoresis of goat anti-chicken IgG antisera. Each well was loaded with 3 μl of sample. Well 1, purified egg extract; well 2, purified chicken IgG (Sigma); well 3, chicken serum; and well 4, pooled egg yolk diluted 1/3 with PBS. 50 μl of goat anti-chicken IgG antiserum was added to each trough. The anode is on the left side of the plate. *B*: immunodiffusion of goat anti-chicken IgG antisera. All wells were loaded with 10 μl of sample. The central well contained goat anti-chicken IgG antiserum. Well 1, chicken IgG; well 2, diluted egg yolk; well 3, purified egg extract; well 4, chicken serum.

poor (Otani et al., 1989). Ovine α-lactalbumin, on SDS-PAGE, has an apparent molecular weight almost identical to that of bovine α-lactalbumin, 14.2 kDa (Whitney et al., 1976), and although we were unable to visualise egg yolk-derived precipitating antibodies we were able to show good immune responses by Western blotting and ELISA. The antibody appeared to be highly specific for α-lactalbumin from whey but cross-reacted with both ovine and bovine α-lactalbumin after Western blotting (Fig. 3B), indicating the presence of common epitopes between the two species. Likewise, Otani et al. (1989) produced egg yolk antibodies to bovine α S1-casein (molecular weight 23.5 kDa), and more recently Lee et al. (1991) successfully used chickens to generate antibodies to human insulin which has a molecular weight of less than 6 kDa. Both these groups used ELISA to monitor antibody response and the presence or otherwise of precipitating antibodies to both antigens was not reported.

As previously acknowledged, Polson et al. (1980) have listed the numerous advantages of using chickens to generate specific antibodies. A number of authors subsequently have focused on the phylogenetic distance between birds and mammals suggesting that this greatly increases the probability of mammalian proteins stimulating an immune response in the serum and egg yolk of inoculated hens (Vieira et al., 1984; Ntakarutimana et al., 1992; Stuart et al., 1988). Gassmann et al. (1990) reported that low levels, 20–30 μg, of a highly conserved mammalian protein, proliferating cell nuclear antigen, induced an immune response in chickens in contrast to their failure to demonstrate specific antibodies in rabbits to the same antigen. They also concluded that increasing the amount of antigen administered to evoke an immune response in chickens was not necessarily accompanied by an increase in the level of response. In this context, a pilot study by us prior to this current investigation suggested that the purity of the antigen used in the inoculation of hens was an important consideration. We found that low levels of contaminating proteins, < 2 μg, was sufficient to induce an immune response in chickens (data not shown), confirming that only low amounts of antigen are required. This finding also highlighted the need to ensure that only the target protein is present in the inoculum. We have found that the excision and elution of the target protein separated on an SDS-PAGE offers a simple and convenient method to generate highly purified antigens (Fig. 3). The ovine α-lactalbumin used in the inoculation schedule (lane 3, Fig. 3A) was prepared from pooled fractions of ovine α-lactalbumin purified by a hydrophobic-interaction chromatographic procedure (lane 2, Fig. 3A). Although the effi-

ciency of the gel excision method in terms of protein recovery is poor (approximately 40%–50%), sufficient highly purified ovine α-lactalbumin antibody may be obtained in a few separations to complete an inoculation schedule employing two animals.

In conclusion, the caprylic acid-ammonium sulphate extraction and purification of chicken IgG from egg yolk has proved to be rapid, simple and reproducible. Although the conditions used were optimised for purification of hyperimmune rabbit serum and not egg yolk, the yield of 136 mg purified IgG per egg is more than sufficient for the needs of an experimental laboratory. Moreover, the extraction procedure is applicable for batch processing with 25 eggs being easily processed up to the shell frozen stage prior to lyophylisation by one person in a single working day. This study further confirmed the advantage of using chickens for the generation of antibodies of high specificity to a mammalian protein such as ovine α-lactalbumin.

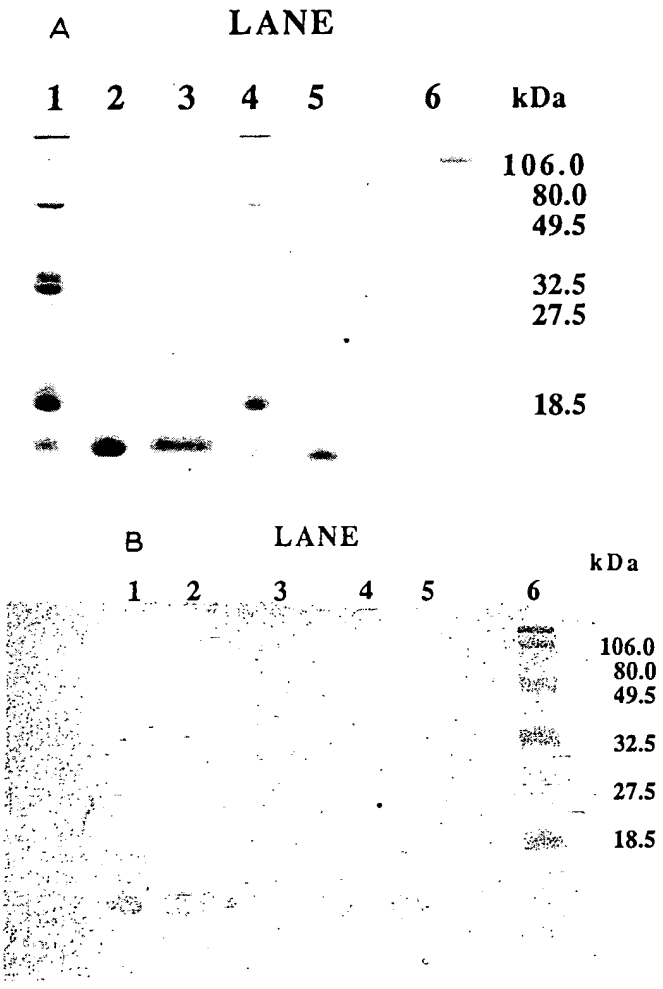

Fig. 3. *A*: SDS-PAGE (15%) of ovine whey, 20 μg, lane 1; pooled fraction of ovine α-lactalbumin from hydrophobic interaction chromatography, 4 μg, lane 2; ovine α-lactalbumin from the gel excision procedure, 4 μg, lane 3; bovine whey, 16 μg, lane 4; bovine α-lactalbumin from Sigma, 4 μg, lane 5; prestained molecular weight markers from Bio-Rad, lane 6. *B*: Western blot of samples described in *A* using chicken anti-ovine α-lactalbumin at a concentration of 1/750. Samples were electrophorectically transferred to nitrocellulose membrane prior to immunodetection.

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3) ~~FESTIN et al., Journal of Immunological Methods Vol 177 (1-2): 215-224 (December 28, 1994)~~.
4) ~~KEENEY et al., CYTOMETRY Vol 34 (2): 61-70 (April 15, 1998)~~.
5) MACEY et al., Journal of Immunological Methods Vol 204 (2): 175-188 (May 26, 1997).
6) McCARTHY et al., Journal of Immunological Methods Vol 163 (2): 155-160 (August 9, 1993).

Thanks a bunch!!!

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Phenotyping analysis of peripheral blood leukocytes in patients with multiple sclerosis

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Multiple sclerosis (MS) is a central nervous disease thought to be elicited by an autoimmune process. Many studies in recent years have concentrated on finding the alterations in the peripheral blood immune profile in MS patients that would reflect disease activity. In the present study, we investigated surface antigen expression on lymphocytes and granulocytes from MS patients and control subjects. We have studied 29 patients suffering from relapsing-remitting or relapsing-progressive forms of MS. The disease was diagnosed in all patients at least 12 months before inclusion into the study. All patients had no attack at the study entry date or within a previous month. The control group included 29 age-matched subjects. Phenotyping of peripheral blood leukocytes was carried out with different fluorescence-conjugated murine monoclonal antibodies. The analysis was performed with three-color flow cytometry. The following antigens were determined [cluster of definition (CD)]: leukocyte common antigen (LCA) (B220, T 200, Ly-5), CD45; LPS-R (lipopolysaccharide receptor), CD14; found on all T cells, CD3; LFA-2 (lymphocyte function associated antigen, T 11), CD2; coreceptor for MHC class II molecules, found on helper T cells, CD4; coreceptor for MHC class I molecules, found on suppressor/cytotoxic T cells, CD8; B4, found on all human B cells, CD19; NCAM (neural cell adhesion molecule), CD56; integrin $\beta 2$ subunit, associated with CD11a (CD11a/CD18, LFA-1, $\alpha L\beta 2$) and CD11b (CD11b/CD18, Mac-1,CR3, $\alpha M\beta 2$), CD18; αL, α subunit of integrin LFA-1 ($\alpha L\beta 2$, CD11a/CD18), CD11a; αM, α subunit of integrin Mac-1 (CR3, $\alpha M\beta 2$, CD11b/CD18), CD11b; ICAM-1 (intercellular adhesion molecule), CD54; H-CAM, Hermes antigen, Pgp-1, CD44; AIM (activation inducer molecule), early activation antigen, CD69; T-cell receptor $\gamma\delta$, TCR $\gamma\delta$. In the MS group, we have found a significant increased expression of CD54 and CD44 antigens on lymphocytes, and higher percentage CD54$^+$ and CD11a$^+$CD54$^+$ lymphocytes out of all lymphocytes compared with the control group. We have also found a significant increased expression of CD11a, CD18 and CD54 antigens on granulocytes, and higher percentage CD11b$^+$CD18$^+$ granulocytes out of all granulocytes in MS patients compared with control. Higher levels of expression of the adhesion molecules may reflect the activation state of leukocytes in MS patients. Eur J Neurol 6:347–352 © 1999 Lippincott Williams & Wilkins

Keywords: multiple sclerosis, peripheral blood lymphocytes, peripheral blood granulocytes, adhesion molecules

INTRODUCTION

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) of putative autoimmune origin (reviewed by Storch and Lassmann, 1997). It is admitted today that to initiate the inflammatory reaction in MS lesions, T cells activated toward self- or cross-reactive antigens need to migrate from the bloodstream into the CNS compartment (reviewed by Hartung *et al.*, 1995). MS plaques are characterized by focal T cells, B cells, macrophages and glial cell infiltration, particularly in the areas around small venules. Factors determining the composition and temporal evolution of the cellular infiltrate in MS brain are unknown (reviewed by Hohlfeld, 1997). The mechanism of crossing the blood–brain barrier and gaining access to the CNS by leukocytes involves adhesive interactions between these cells and cerebral microvascular endothelial cells. Inflammatory reactions that comprise leukocyte activation and transmigration appear to be controlled by numerous cytokines and adhesion molecules (reviewed by Olsson *et al.*, 1990;

Springer, 1994; Hafler and Weiner, 1995). The extravasation of leukocytes into the CNS parenchyma is facilitated by the expression of adhesion molecules on both leukocytes and cerebral vascular endothelial cells (reviewed by Svenningsson *et al.*, 1993; Droogan *et al.*, 1996; Karlik *et al.*, 1997).

The evidence supporting a systemic immune abnormality in MS includes a decrease in a functional suppressor activity of circulating lymphocytes, abnormalities of effector cell functions, defects in interferon production by peripheral blood lymphocytes and abnormal numbers of circulating lymphocyte subsets determined by immunophenotyping (reviewed by Bansil *et al.*, 1994; Hunter and Rodriguez, 1995). It is not clear whether these immune aberrations in MS represent a primary defect and contribute to the disease pathogenesis directly or represent a secondary event.

The development of immunological techniques, mostly by means of monoclonal antibodies, has allowed the study of cell surface markers and the understanding of the role of leukocyte subsets in immune regulation. In MS, the abnormalities in peripheral blood immunophenotypes have been found in different forms and phases of disease activity. No characteristic patterns for MS have so far been identified.

In our study, three-color flow cytometry was applied to search for distinctive populations of peripheral blood leukocyte immunophenotypes in patients with relapsing-remitting or relapsing-progressive forms of MS and with mild or moderate disability. We have also included the analysis of leukocyte adhesion molecules due to their putative pathogenic and therapeutic implications.

METHODS

Patients

We have studied 29 out-patients (21 women and eight men) with clinically definite or laboratory-supported definite MS (Poser *et al.*, 1983). The mean age was 32.6 years [95% confidence interval (CI) 29.8- 35.4]. The diagnosis was based on the history, clinical signs and results of laboratory methods (magnetic resonance imaging, cerebrospinal fluid (CSF) immunoglobulin (Ig)G oligoclonal bands, and visual evoked potentials). Twenty-two patients had relapsing-remitting form and seven had relapsing-progressive form of MS. The disease was diagnosed in all patients at least 12 months before inclusion into the study and the mean disease duration was 4.3 years (95% CI 3.1 - 5.5). All patients had no attack at the study entry date or within the previous month. The mean Kurtzke's expanded disability status scale (Kurtzke, 1983) score was 3.2 (95% CI 2.9 3.5). No patient had more than 5.5 scores.

None of the patients had received corticosteroid treatment for at least 4 weeks preceding the investigation. The cytostatic or immunomodulatory therapy had never been applied to them. No patient suffered from other neurological disease.

Control group

The control group included 29 age-matched subjects (20 healthy volunteers and nine patients with other neurological diseases epilepsy, headache, neuralgia, discopathy). There were 20 women and nine men. The mean age was 31.5 years (95% CI 28.1 34.8).

Cell preparations and flow cytometry analysis

All blood samples were obtained from patients consulted in the 2nd Department of Neurology, Institute of Psychiatry and Neurology. The study protocol was approved by the local Ethics Committee.

Two milliliters of venous blood samples from MS patients and control subjects were collected into a tube containing heparin. Phenotyping was carried out with a panel of different fluorescence-conjugated mouse monoclonal antibodies (mAb). For a color separation, each panel contained mAb conjugated with fluorescein isothiocyanate (FITC), R-phycoerythrin (RPE) or fluorochrome consisting of the cyanin-5 (Cy-5) covalent coupled to R-phycoerythrin (RPE-Cy5).

According to the manufacturers' established procedures, an adequate volume of specific mAb was mixed with aliquots of whole blood samples containing 5×10^5 cells. The following anti-human mAb obtained from the suppliers (given in parentheses) were used: anti-CD18 (LFA-1, Beta-Chain) conjugated with FITC (DAKO A/S, Glostrup, Denmark), anti-CD11b (C3bi receptor) conjugated with RPE (DAKO), anti-CD3RPE-Cy5 (T Cell) conjugated with RPE-Cy5 (DAKO), anti-CD11a (LFA-1, Alpha-Chain) conjugated with FITC (DAKO), anti-CD54 (Leu-54) conjugated with PE (Becton Dickinson Immunocytometry System, San Jose, CA, USA), anti-CD44 (Leu-44) conjugated with FITC (Becton Dickinson), anti-TCR-γ/δ-1 conjugated with PE (Becton Dickinson), anti-CD69 (Leu-23) conjugated with FITC (Becton Dickinson), anti-CD56 (Leu-19) conjugated with PE (Becton Dickinson), anti-CD8 conjugated with FITC, anti-CD4 conjugated with RPE; anti-CD2 conjugated with FITC, anti-CD19 conjugated with RPE (DAKO Dual Color Reagent); anti-CD45 conjugated with FITC, anti-CD14 conjugated with RPE (DAKO Dual Color Reagent). For identification of the different CDs, see definitions presented in the abstract (Ager *et al.*, 1997).

The cell suspensions were incubated in the dark at 4°C for 30 min. The red cells were then lysed by adding to each test tube 4 ml of Ortho-mune Lysing

Reagent (Ortho Diagnostic Systems, Neckargemünd, Germany). When the lysis was complete, the leukocytes were collected by centrifugation at 400 × *g* for 6 min at 4°C and the supernate was discarded. Then white blood cells were washed by adding 2 ml buffer containing phosphate buffered saline with 1% bovine serum albumin and 0.1% sodium azide (PBS–BSA) to each test tube and centrifuged at 400 × *g* for 6 min at 4°C, and the supernate was discarded. The wash procedure was repeated with 1 ml PBS BSA. The cells were resuspended in an appropriate fluid (1% paraformaldehyde in PBS) for flow cytometry analysis.

IgG1 conjugated with FITC/IgG2a conjugated with PE (Simultest Control $\gamma 1/\gamma 2a$, Becton Dickinson) and mouse IgG1 conjugated with FITC/IgG1 conjugated with RPE/IgG1 conjugated with RPE-Cy5 (DAKO Triple Color Reagent) were used as a negative control.

The analysis was performed with three-color flow cytometry using Cytoron-Absolute Ortho Diagnostic. Sample data were analysed by Immunocant II software.

Results were expressed as percentages of each subset to respectively gated lymphocytes or granulocytes. The intensity of fluorescence was performed as mean fluorescence channel (mch) and relative fluorescence index (RFI). The RFI was calculated as a quotient of mean fluorescence channel cells stained with mAb conjugated with FITC or RPE and mean channel cells stained with respective (according to isotype) negative control.

Statistical evaluation

If data were found to be normally distributed, the comparison between control and MS groups was performed using Student's *t*-test. The non-parametric Mann–Whitney U-test was used to compare the groups in case data were not found to be normally distributed. *P* values equal to or lower than 0.05 were considered statistically significant.

RESULTS

Lymphocytes

The percentage of $CD54^+$ lymphocytes out of all lymphocytes was significantly higher ($P = 0.03$) in MS patients than in controls. We have also found increased expression of CD54 antigen (mch, $P < 0.000001$) on lymphocytes in the MS group compared with the control group. MS patients had higher percentage of $CD11a^+CD54^+$ lymphocytes, out of all lymphocytes, than the controls ($P = 0.04$). The significant increased expression of CD44 antigen (mch, $P = 0.005$; RFI, $P = 0.01$) on lymphocytes was also found in MS patients compared with the control group (Table 1).

We have not found any significant differences between MS patients and the control group in percentage of $CD2^+$, $CD2^+CD3^+$, $CD3^+$, $CD4^+CD3^+$, $CD8^+CD3^+$, $CD11a^+$, $CD11a^+CD3^+$, $CD11b^+$, $CD11b^+CD3^+$, $CD11b^+CD18^+$, $CD18^+$, $CD18^+CD3^+$, $CD19^+$, $CD44^+$, $CD44^+CD3^+$, $CD54^+CD3^+$, $CD56^+$, $CD56^+CD3^+$, $\gamma\delta^+$TCR, $\gamma\delta^+$TCRCD3$^+$, $CD44^+\gamma\delta^+$TCR, $CD69^+$, $CD69^+CD3^+$, $CD69^+CD56^+$ lymphocytes out of all lymphocytes. The expression (mch and RFI) of CD11a, CD11b and CD18 antigens on lymphocytes did not differ significantly in MS patients and controls (Table 2).

TABLE 1. Adhesion molecules on lymphocytes in MS patients and the control group

Type	MS patients ($n = 28$)	Controls ($n = 29$)	*P* level[d]
$CD54^+$[a]	9.60 (± 2.60)	8.31 (± 2.66)	0.03
$CD54^+$[b]	85.02 (± 4.53)	77.53 (± 2.96)	0.000001
$CD11a^+$ $CD54^+$[a]	8.88 (± 2.49)	7.65 (± 2.93)	0.04
$CD44^+$[b]	86.57 (± 7.60)	81.76 (± 7.10)	0.005
$CD44^+$[c]	3.08 (± 0.27)	2.89 (± 0.31)	0.01

Results are presented as mean ± SD.
[a]Percentage (%) lymphocytes out of all lymphocytes.
[b]Mean fluorescence channel (mch).
[c]Relative fluorescence index (RFI).
[d]Mann–Whitney U test.

Granulocytes

We have found significant increased expression of CD11a (mch, $P = 0.002$; RFI, $P = 0.002$), CD18 (RFI, $P = 0.005$) and CD54 (mch, $P = 0.02$) antigens on granulocytes in the MS group compared with controls. The percentage of $CD11b^+CD18^+$ granulocytes out of all granulocytes in MS patients was significantly higher compared with the control group ($P = 0.03$) (Table 3).

We have found no statistical differences in the percentage of $CD11a^+$, $CD11a^+CD54^+$, $CD11b^+$, $CD18^+$, $CD54^+$ granulocytes out of all granulocytes between MS and control groups. There was no significance in expression of CD11b and CD14 antigens on granulocytes (Table 4).

DISCUSSION

In the three-color flow cytometry analysis of surface molecule expression by peripheral blood lymphocyte subsets, we have found a significantly higher percentage of $CD54^+$ lymphocytes out of all lymphocytes, and increased expression of this antigen on lymphocytes in the MS group as revealed by mch compared with control subjects. Antigen CD54 is the immunoglobin superfamily member that is expressed on various cells, such as vascular endothelial cells, astrocytes, B cells, T

TABLE 2. Phenotypes of peripheral blood lymphocytes, which did not significantly differ in MS patients and the control group

Type	MS patients (n = 28)	Controls (n = 29)
CD2+[a]	80.62 (± 5.98)	79.11 (± 4.47)
CD2+ CD3+[a]	68.61 (± 8.89)	69.07 (± 6.68)
CD3+[a]	70.72 (± 7.41)	72.18 (± 6.07)
CD4+ CD3+[a]	47.84 (± 8.18)	48.03 (± 6.18)
CD8+ CD3+[a]	16.40 (± 5.10)	17.23 (± 5.64)
CD11a+[a]	97.43 (± 1.81)	97.37 (± 1.49)
CD11a+[b]	132.16 (± 8.57)	128.41 (± 6.93)
CD11a+[c]	4.73 (± 0.42)	4.56 (± 0.51)
CD11a+ CD3+[a]	70.81 (± 7.51)	71.94 (± 6.00)
CD11b+[a]	31.49 (± 7.32)	32.49 (± 6.03)
CD11b+[b]	131.04 (± 6.63)	128.17 (± 7.29)
CD11b+[c]	26.34 (± 6.53)	29.57 (± 8.95)
CD11b+ CD3+[a]	10.08 (± 5.23)	12.93 (± 6.54)
CD11b+ CD18+[a]	30.86 (± 7.50)	31.48 (± 7.45)
CD18+[a]	96.95 (± 2.65)	96.96 (± 1.52)
CD18+[b]	127.56 (± 8.19)	124.03 (± 6.77)
CD18+[c]	4.57 (± 0.44)	4.40 (± 0.47)
CD18+ CD3+[a]	70.54 (± 7.52)	72.14 (± 5.95)
CD19+[a]	11.43 (± 3.53)	11.86 (± 3.27)
CD44+[a]	46.90 (± 23.82)	48.56 (± 23.20)
CD44+ CD3+[a]	36.67 (± 21.00)	40.35 (± 20.53)
CD54+ CD3+[a]	0.75 (± 0.81)	0.99 (± 0.84)
CD56+[a]	17.19 (± 7.55)	16.66 (± 5.10)
CD56+ CD3+[a]	3.25 (± 2.51)	4.03 (± 3.43)
γδ+ TCR[a]	4.55 (± 2.84)	4.67 (± 2.84)
γδ+ TCR CD3+[a]	4.33 (± 2.86)	4.43 (± 2.84)
CD44+ γδ+ TCR[a]	0.73 (± 0.73)	0.89 (± 1.39)
CD69+[a]	1.80 (± 1.15)	2.25 (± 1.37)
CD69+ CD3+[a]	0.54 (± 0.29)	0.61 (± 0.24)
CD69+ CD56+[a]	0.55 (± 0.28)	0.43 (± 0.19)

Results are presented as mean ± SD.
[a]Percentage (%) lymphocytes out of all lymphocytes.
[b]Mean fluorescence channel (mch).
[c]Relative fluorescence index (RFI).

TABLE 3. Adhesion molecules on granulocytes in MS patients and control group

Type	MS patients (n = 25)	Controls (n = 28)	P level[d]
CD11a+[a]	117.71 (± 5.97)	113.52 (± 3.33)	0.002
CD11a+[b]	2.45 (± 0.19)	2.26 (± 0.20)	0.002
CD18+[b]	2.79 (± 0.25)	2.58 (± 0.26)	0.005
CD54+[a]	94.43 (± 20.55)	78.59 (± 7.44)	0.02
CD11b+ CD18+[c]	99.79 (± 0.18)	99.46 (± 0.76)	0.03

Results are presented as mean ± SD.
[a]Mean fluorescence channel (mch).
[b]Relative fluorescence index (RFI).
[c]Percentage (%) granulocytes out of all granulocytes.
[d]Mann–Whitney U test.

TABLE 4. Phenotypes of peripheral blood granulocytes, which did not significantly differ in MS patients and the control group

Type	MS patients (n = 25)	Controls (n = 28)
CD11a+[a]	99.33 (± 0.79)	99.58 (± 0.59)
CD11a+ CD54+[a]	5.97 (± 6.69)	10.05 (± 12.17)
CD11b+[a]	99.84 (± 0.19)	99.59 (± 0.67)
CD11b+[b]	182.91 (± 13.13)	180.48 (± 15.63)
CD11b+[c]	14.81 (± 2.63)	16.85 (± 4.59)
CD14+[b]	96.74 (± 10.05)	91.80 (± 7.39)
CD14+[c]	7.97 (± 1.44)	8.65 (± 1.84)
CD18+[a]	99.84 (± 0.16)	99.57 (± 0.68)
CD18+[b]	133.63 (± 7.72)	129.42 (± 7.19)
CD54+[a]	6.40 (± 6.89)	10.08 (± 12.17)

Results are presented as mean ± SD.
[a]Percentage (%) granulocytes out of all granulocytes.
[b]Mean fluorescence channel (mch).
[c]Relative fluorescence index (RFI).

cells and macrophages. Resting T cells express little or no CD54, while activated T cells express significant amounts of this antigen (Van den Noort *et al.*, 1989; Raine and Cannela, 1992). The increased expression of CD54 on T cells from patients with MS might play an important role in the enhancement of the antigen recognition, leading to effector functions that may be responsible for the immunopathogenesis of MS.

We have also found the significant increase of CD44 antigen expression on lymphocytes in MS patients compared with control subjects. The higher expression of this antigen on CSF T lymphocytes, but not on peripheral blood lymphocytes, has been reported (Girgrah *et al.*, 1991). The CD44 antigen is a proteoglycan implicated in several adhesion events including the initial adhesion of T cells to endothelium (Antel and Owens, 1993). Our observation that there is higher expression of adhesion molecules CD54 and CD44 in MS patients may reflect the general activation of peripheral blood lymphocytes. However, from our findings we cannot define whether the altered expression of the above-mentioned antigens represents a primary defect and contributes to the disease pathogenesis directly, or represents a secondary event. This may result from a response to an activating signal, such as lymphokines released by cells participating in inflammatory reactions in the CNS (reviewed by Hunter and Rodriguez, 1995).

In our study, we have found that MS patients had a higher percentage of $CD11a^+CD54^+$ lymphocytes out of all lymphocytes than the control group. This constitutes further evidence of the peripheral immune activation.

Our results did not confirm those reported by some

authors of alterations in the percentage of $CD2^+$, $CD3^+$, $CD19^+$, $CD56^+$, $\gamma\delta^+$ TCR and $CD69^+$ lymphocytes (Kreutzfelder *et al.*, 1992; Munschauer *et al.*, 1995; Bongioanni *et al.*, 1996). One reason for these differences may lie in the various methods (staining by fluorescent or enzyme-linked antibodies, type of antibody used, enumeration of the cells by microscopic evaluation or by flow cytometry, using one-, two- or three-color flow cytometry) used to determine the peripheral blood cells. It also might result from a disease phase during which the blood samples were collected.

We have not found significant difference in the peripheral blood $CD8^+$ T lymphocytes (suppressor/cytotoxic) between the MS group and controls. Many studies on lymphocyte subsets in MS have revealed a loss of circulating $CD8^+$T cells (Huddlestone and Oldstone, 1979; Compston, 1983; Thompson *et al.*, 1985; Kreutzfelder *et al.*, 1992; Crucian *et al.*, 1995). However, these T-cell changes were found to be related to increased disease activity, as patients in remission or with inactive disease have normal numbers of suppressor/cytotoxic cells (Kastrukoff and Paty, 1984; Rose *et al.*, 1988; Capra *et al.*, 1992). These reports may explain our findings. It should be also pointed out that some studies (Hirsch *et al.*, 1985; Frequin *et al.*, 1993; Calopa *et al.*, 1995) do not confirm reduction in $CD8^+$ cell numbers. A possible source of controversy is the poor correlation between the activity of the pathological process and the clinical findings.

Three-color flow cytometry analysis of peripheral blood granulocytes revealed a significantly increased expression of CD11a, CD18 and CD54 antigens in the MS group compared with the control group. Also, the percentage of $CD11b^+CD18^+$ granulocytes out of all granulocytes in MS patients was significantly higher than in controls. There are relatively few available data addressing the role of granulocytes in MS pathogenesis. The granulocytes participate mainly in a nonspecific immune response, as they have no capacity for specific antigen recognition. Nevertheless, granulocytes cooperate with a specific response. The chemotactic factors (i.e. TNF-β) can mobilize granulocytes to a place of an initiating immune or inflammatory response. The hypothesis has been recently set forth that activated granulocytes are suited to modify self-proteins (Ludewig, 1993). Modified self-proteins may sensitize the immune system or break the immune tolerance, which can result in an autoimmune reaction. Thus, granulocytes not only effectively eliminate pathogens, but damage tissues of the host as well. Our results may indicate the presence of activated granulocytes in peripheral blood, which can initiate the cascade of events leading to the injury of CNS. CD11b/CD18 is a leukocyte integrin that plays a critical role in neutrophil adhesion and the initiation of acute inflammatory responses (Violette *et al.*, 1995). On the other hand, it cannot be ruled out that an increased expression adhesion molecule, especially CD54 antigen on granulocytes and higher percentage of $CD11b^+CD18^+$ granulocytes in MS patients, is only a consequence of T-cell stimulation. It is known that stimulated T cells generating various lymphokines may participate in activation of granulocytes. Neutrophil–endothelial cell interactions, making transmigration into tissues possible, are mediated by interacting sets of cell adhesion molecules. The firm adhesion of neutrophils to a vessel wall occurs via the interaction of CD11b/CD18 integrin to endithelial ligands such as CD54. This binding requires an activation of CD11b/CD18 by the neutrophil exposure to a variety of activating factors, for instance interleukin-8 (Albelda *et al.*,1994). CD11b/CD18 is involved in eosinophil endothelial adhesion through its counterligand, CD54 (Horie *et al.*, 1997). CD54 is also induced on eosinophils by cytokine stimulation.

The results reported here indicate the presence of immune alterations in peripheral blood of MS patients; however, further studies are essential to clarify the relationship between these changes and a clinical course of MS. More knowledge about adhesion molecules will provide an insight into mechanisms underlying immune responses and an opportunity to manipulate these responses. There is great need to establish the utility of measuring levels of adhesion molecule expression on peripheral blood leukocytes as a means to monitor the immune reactivity and the effect of therapy in MS patients.

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1) HUBL et al., CYTOMETRY Vol 30 (2): 72-84 (April 15, 1997).

2) COWLAND et al., Journal of Immunological Methods Vol 232 (1-2): 191-200.

3) FESTIN et al., Journal of Immunological Methods Vol 177 (1-2): 215-224 (December 28, 1994).

4) KEENEY et al., CYTOMETRY Vol 34 (2): 61-70 (April 15, 1998).

5) MACEY et al., Journal of Immunological Methods Vol 204 (2): 175-188 (May 26, 1997).

6) McCARTHY et al., Journal of Immunological Methods Vol 163 (2): 155-160 (August 9, 1993).

Thanks a bunch!!!

Gail Gabel
7B15
305-0807
ASN 09/388,899

Cytometry (Communications in Clinical Cytometry) 34:61–70 (1998)

Original Articles

Single Platform Flow Cytometric Absolute CD34+ Cell Counts Based on the ISHAGE Guidelines

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In concert with the International Society of Hematotherapy and Graft Engineering (ISHAGE), we previously described a set of guidelines for detection of CD34+ cells based on a four-parameter flow cytometry method (CD45 FITC/CD34 PE staining, side and forward angle light scatter). With this procedure, an absolute CD34+ count is generated by incorporating the leukocyte count from an automated hematology analyser (two-platform method). In the present study, we modified the basic ISHAGE method with the addition of a known number of Flow-Count™ fluorospheres. To reduce errors inherent to sample washing/centrifugation, we implemented ammonium chloride lyse, no-wash no-fix sample processing. These modifications convert the basic protocol into a single-platform method to determine the absolute CD34 count directly from a flow cytometer and form the basis of the Stem-Kit from Coulter/Immunotech. A total of 72 samples of peripheral blood, apheresis packs, and cord blood were analysed and compared using the ISHAGE protocol with or without the addition of fluorescent microspheres. Comparison of methods showed a high correlation coefficient ($r = 0.99$), with no statistically significant difference or bias between methods ($P > 0.05$). Linearity of the absolute counting method generated an R^2 value of 1.00 over the range of 0–250/μl. Precision of the absolute counting method measured at three concentrations of CD34+-stabilised KG1a cells (Stem-Trol, COULTER®) generated a coefficient of variation (C.V.) ranging from 4% to 9.9%. In a further modification of the single-platform method, the viability dye 7-amino actinomycin D was included and demonstrated that both viable and nonviable CD34+ cells could be identified and quantitated. Together, these modifications combine the accuracy and sensitivity of the original ISHAGE method with the ability to produce an absolute count of viable CD34+ cells. It is the accurate determination of this value that is most clinically relevant in the transplant setting. These modifications may improve the interlaboratory reproducibility of CD34 determinations due to the reduction in sample handling and calculation of results. Cytometry (Comm. Clin. Cytometry) 34:61–70, 1998. © 1998 Wiley-Liss, Inc.

Key terms: ISHAGE guidelines; CD34; 7-AAD; absolute counts

The CD34 antigen is expressed on 2–4% of normal marrow mononuclear cells, and these cells exhibit multilineage progenitor cell activity in vitro (1–4). Landmark studies performed by Berenson et al. (5) demonstrated that CD34+ bone marrow cells could reconstitute long-term multilineage hematopoiesis in nonhuman primates. More importantly, it established for the first time that the most primitive hematopoietic stem cells are contained within the CD34+ fraction. Subsequent human studies in patients treated with myeloablative chemotherapy have confirmed these data (6).

In normal individuals, low numbers of CD34+ cells can be identified in the peripheral blood. These cells can be "mobilised" from the marrow into the blood in significantly higher numbers by chemotherapy (7) and/or recombinant cytokines (8), and at the present time mobilised peripheral blood stem cell (PBSC) are the preferred alternative to marrow in the autologous transplant setting (9). With this increased use of PBSCs, it has become apparent that rapid, sensitive, and reproducible methodologies are required for the accurate assessment of the engraftment potential of such collections.

At present, the quantitation of CD34 antigen-positive cells by flow cytometry is the preferred method for assessing graft adequacy of PBSC collections. Because the procedure can be performed in less than 1 h, it is also suitable for optimising the timing of apheresis collections and for the "real-time" analysis of such collections. Unfor-

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tunately, although a number of flow cytometry assays for CD34 enumeration have been described (10–15), the lack of a standardised method has led to the generation of divergent data (16–18). To address this issue, we have, in concert with the International Society of Hematotherapy and Graft Engineering (ISHAGE), designed a set of guidelines (19) for the accurate detection of CD34+ cells based on our previously described four-parameter flow cytometry method (CD45 FITC/CD34 PE staining, side and forward angle light scatter; FALS) (20). This procedure is very sensitive, being capable of detecting 10–20 CD34+ cells per 100,000 CD45+ nucleated white blood cells. It is highly specific, using appropriate pan-CD45 antibodies (that detect all isoforms and glycoforms) and CD34 conjugates (that detect all CD34 glycoforms), is quick and simple, and can be performed on a variety of single-laser flow cytometers with only basic software being required for data analysis.

In the present study, we modified the ISHAGE protocol by including a known number of fluorescent microspheres, thus converting the flow cytometer into a single-instrument absolute CD34+ cell-counting device. In a further development aimed at increasing the accuracy of the basic method, we incorporated a simple ammonium chloride-based lysis step that obviates the requirement for washing/centrifugation steps and the use of fixatives. In a separate experiment, we incorporated a viability dye into the protocol to determine the absolute viable CD34+ cell count, arguably the most clinically relevant parameter in the assessment of graft adequacy.

MATERIALS AND METHODS

Study Design

Comparison of single platform and dual-platform ISHAGE methodology. Over a 5-month period, consecutive samples from patients at the London Health Sciences Centre undergoing peripheral stem cell harvests after mobilisation for 5 d with G-CSF (5 μg/kg) were analysed for CD34+ cells by flow cytometry. A total of 72 samples of peripheral blood (n = 35), apheresis packs (n = 31), or cord blood (n = 6) were studied. All samples were diluted to a leukocyte count of less than 20 × 10^9/l when necessary. Samples were analysed by the basic ISHAGE protocol (two-platform method) and the modified single-platform ISHAGE method with the addition of a fluorescent bead (Stem-Kit, described below).

Linearity and precision study. Linearity of the absolute counting method was determined by doubling dilutions of Stem-Trol KG1a cells in COULTER® Cyto-Trol™ control cells in the range of 0–250/μl. Precision of the absolute counting method was measured at three target points (approximately 25, 125, and 200 CD34+ cells/μl) by replicate analysis of Stem-Trol KG1a cells in normal whole blood. For each level, the same sample was analysed eight times.

Incorporation of a cell viability stain. In a separate experiment, the viability dye 7-amino-actinomycin D (7-AAD; Molecular Probes, Eugene, OR) was added at a concentration of 1 μg/ml to a fresh and aged apheresis sample. This dye has been shown to be useful in the discrimination of live versus dead cells (21).

Statistics

Comparison between results for absolute number of CD34+ cells using basic ISHAGE and the modified single-platform ISHAGE protocol with Stem-Kit was done by using Spearman correlation and the Wilcoxon signed rank test for nonparametrically distributed data (SigmaStat, Jandel Scientific, San Rafael, CA). A bias plot (22) was also used for method comparison.

Antibodies and Kits

Basic ISHAGE method. For samples analysed with the basic ISHAGE method, cells were stained with a fluorescein isothiocyanate (FITC) conjugate of a CD45 antibody (clone J33, Coulter/Immunotech) (23) that detects all isoforms and glycoforms of the CD45 antigen family (19), and a R-phycoerythrin (RPE) conjugate of a CD34 antibody (clone 581, Coulter/Immunotech, Marseilles, France) (24) that detects a class III epitope on all glycoforms of the CD34 antigen (4,19). Control samples were stained with CD45 FITC and an IgG1PE isotype. All antibodies were used at the manufacturer's recommended concentration after verification of reactivity in house.

Stem-Kit. The Coulter/Immunotech Stem-Kit is designed to identify CD34+ cells by using ISHAGE guidelines criteria (19). The prototype kit used in the present study contains CD34 PE (clone 518) and CD45-FITC (clone J33) monoclonal antibodies in a single vial and Flow-Count fluorospheres for determination of the absolute count of CD34+ cells in human blood and blood-derived samples by single-instrument-platform flow cytometry. In this modification of the basic ISHAGE method, a known volume and accurately assayed concentration of Flow-Count fluorospheres are added to an equal volume of the patient's blood sample. This establishes a ratio of fluorospheres to volume of sample. Because the concentration of the fluorospheres is known, the absolute count of the CD34+ cells can be calculated. The Stem-Kit also contains a novel CD45-FITC/CD34 (ISOCLONIC) control to enumerate nonspecifically stained events. In this control, unconjugated CD34 antibody is present in large excess to block specific staining of PE-labelled CD34 present at the same concentration as the test (12). In addition, Stem-Kit also includes Stem-Trol Control Cells. These are KG1a cells that have been modified to present the 581/CD34 and J33/CD45 epitopes at densities similar to those found on normal CD34+ hematopoietic cells (25).

Sample Preparation

Basic ISHAGE method. For each patient sample, four 12- × 75-mm tubes were labelled; 45/34 (nos. 1 and 2), WASH (no. 3), and 45/IgG (no. 4). Two millitres of Dulbecco's phosphate-buffered saline (DPBS; Gibco BRL, Grand Island, NY) were added to the WASH tube, and exactly 100 μl of the well-mixed blood/apheresis sample were added to the bottom of the other tubes by using a repeater pipette (Eppendorf 4780, Brinkmann Instruments

Ltd., Mississauga, Canada). Leukocyte counts were determined on a Coulter STKS hematology analyser. If necessary, samples were diluted in DPBS to attain a leukocyte count (LKC) of $<20 \times 10^9$/l. Ten microlitres of CD45 FITC and 10 µl CD34 PE were added to the first two tubes. Ten microlitres of CD45 FITC and 10 µL PE IgG1 isotype control were added to the fourth tube. All tubes were incubated at room temperature for 15 min in the dark and then 2 ml ammonium chloride (Ortho-mune, Ortho Diagnostic Systems, Markham, Ontario) were added to lyse red blood cells. All tubes were gently vortexed and incubated at room temperature for 10 min in the dark, washed twice in DPBS (5 min at 500*g*), and resuspended in 1 ml DPBS. Samples were then stored on ice at 4°C in the dark and analysed by flow cytometry within 1 h.

Stem-Kit. For the absolute counting method, samples were prepared exactly as described in the previous section for tubes 1 and 2, with the exception that 20 µl of CD45 FITC/34PE combined reagent were added. For the control tube (no. 4), 20 µl of CD45 FITC/ISOCLONIC control reagent were added to the cells. After ammonium chloride lysis, the cells were not washed; instead, 100 µl of COULTER Flow-Count Fluorospheres were added to tubes 1, 2, and 4 (using the same repeater pipette used for sample aliquoting). All samples were kept in the dark until the time of analysis (always within 1 h) and were gently vortexed immediately prior to analysis.

Gating Strategy

Basic ISHAGE protocol (Fig. 1a,b). Flow cytometric analysis was performed on a Coulter EPICS® XL-MCL. Alignment and calibration was performed daily by using Coulter Flow-Check™ and Flow-Set™ fluorospheres. Compensation was checked visually with each run and adjusted by using Coulter CYTO-COMP™ cells when required. Seventy-five thousand CD45+ events were collected on histogram 2, with a minimum count of 100 events in region (R) 4 on histogram 4. Region 5 on histogram 1 was set precisely to include only lymphocytes (bright CD45, low side scatter) and displayed on histogram 6 (FALS vs. side scatter). This helps to establish the minimum size range for the lymph-blast region (R4, histogram 6). Events such as platelets and other nonspecifically stained debris, if present, can be excluded. This region also helps to confirm that the FALS discriminator and FALS detector volts/gain are adequately set. The discriminator (or forward scatter threshold) was set to ensure that even the smallest CD45+ lymphocytes scattered above it. FALS volts/gain were adjusted so that the smallest lymphocytes scattered around channel 200 of a 1,024 × 1,024 linear dot histogram. After determining the appropriate discriminator setting, R1 was positioned on histogram 1 to include all CD45+ events. The lower extremity of R1 was set low enough to include all dim CD45+ events (using histogram 5 as a guide). Histogram 5 (CD34-PE vs. CD45-FITC staining) helps to establish the lower limit of CD45 expression such that potential CD34+ cells (that express low levels of CD45) are not excluded. Histogram 2 was gated on R1. Region 2 was adjusted to include all dim and bright CD34+ events with low/intermediate side scatter. Histogram 3 was gated on R1 and R2. It is on this histogram that a cluster of cells appear, characterised by dim CD45 staining relative to lymphocytes and low side scatter. Region 3 was created as an amorphous region to best delineate the true CD34+ cells from other events. Histogram 4 is gated on the cumulative regions 1-3. The number of events in R4 is usually similar to that found in R3, but, if present, small platelet aggregates that stain weakly with both CD45 and CD34 (and are thus included in R3) can be excluded from R4 on the basis of light scatter. Both test (Fig. 1a) and control (Fig. 1b) samples were analysed. A DPBS blank (tube 3) was run between the duplicate test (tubes 1 and 2) and the control tube (tube 4). The result from the two test samples was averaged, and the number of control IgG PE events was subtracted from the average CD34+ events. For apheresis packs, this result was multiplied by the LKC and the pack volume in litres to give absolute CD34+ cells $\times 10^6$/pack.

Basic ISHAGE protocol calculations.

$$\frac{[\text{[average \#CD34+ (R4)]}] - \text{Isotype control (R4)}}{[\text{(average CD45+ Events Counted (R1)}]} \times \text{LKC } (\times 10^9/\text{L}) \times 1000$$

$$\text{CD34+} = \_\_ \times 10^6/\text{L}$$

Coulter® Stem-Kit analysis (Fig. 2a,b). Histograms 1-6 were done with the basic ISHAGE method. Histogram 7 displays Flow-Count beads on FL3 versus time. Region 6 is set to include only single-bead events, as recommended by the manufacturer. FL3 high voltage should be adjusted to ensure that both single- and double-bead populations are visible. On the Coulter EPICS XL-MCL, R6 is defined as a CAL region to allow automatic calculation of absolute numbers of CD34+ cells.

Histogram 8 displays Flow-Count beads versus forward scatter, and an amorphous R7 is drawn to include all Flow-Count beads. By gating histogram 1 as all events "-R7" on a Coulter XL instrument, a calculation of absolute LKC (CD45+ events) can be derived from R1. On Becton Dickinson FACS instruments, histogram 1 can similarly be displayed as all events minus R7 events (gate 1 = not R7 and R1), and the gate statistics for gate 1 (G1) displays total CD45+ events. The result from the two test samples are averaged, and the number of events in R4 stained with the isoclonic control is subtracted from the average CD34+ events.

Coulter Stem-Kit: Calculation of Absolute CD34+ Cells

For blood or cord samples that have not been diluted, the absolute count can be taken directly from R4 on a Coulter EPICS XL/XL-MCL flow cytometer. The absolute count is expressed as number of cells $\times 10^6$/l. For instruments that do not automatically calculate the CD34

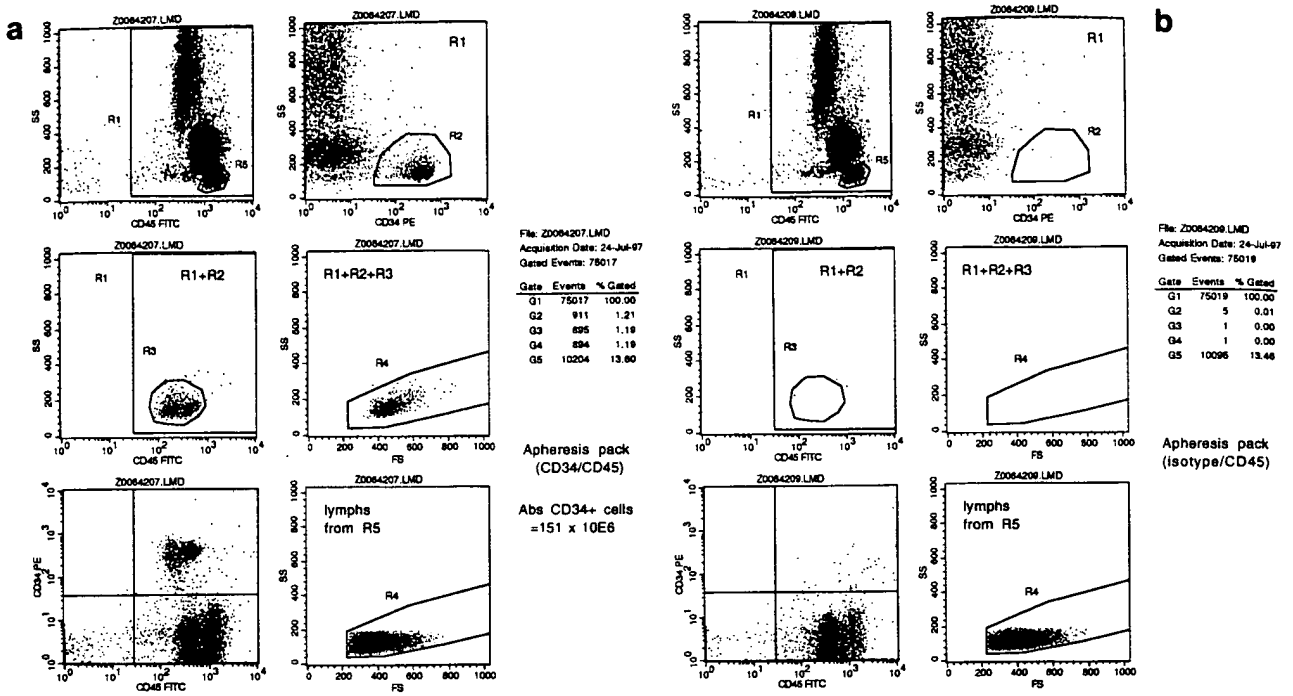

FIG. 1. Basic ISHAGE gating strategy on an apheresis sample (**a**) CD45 FITC/34 PE and (**b**) CD45 FITC/IgG1 PE. Listmode data collected on a Coulter EPICS XL were analysed with Cellquest software (Becton Dickinson).

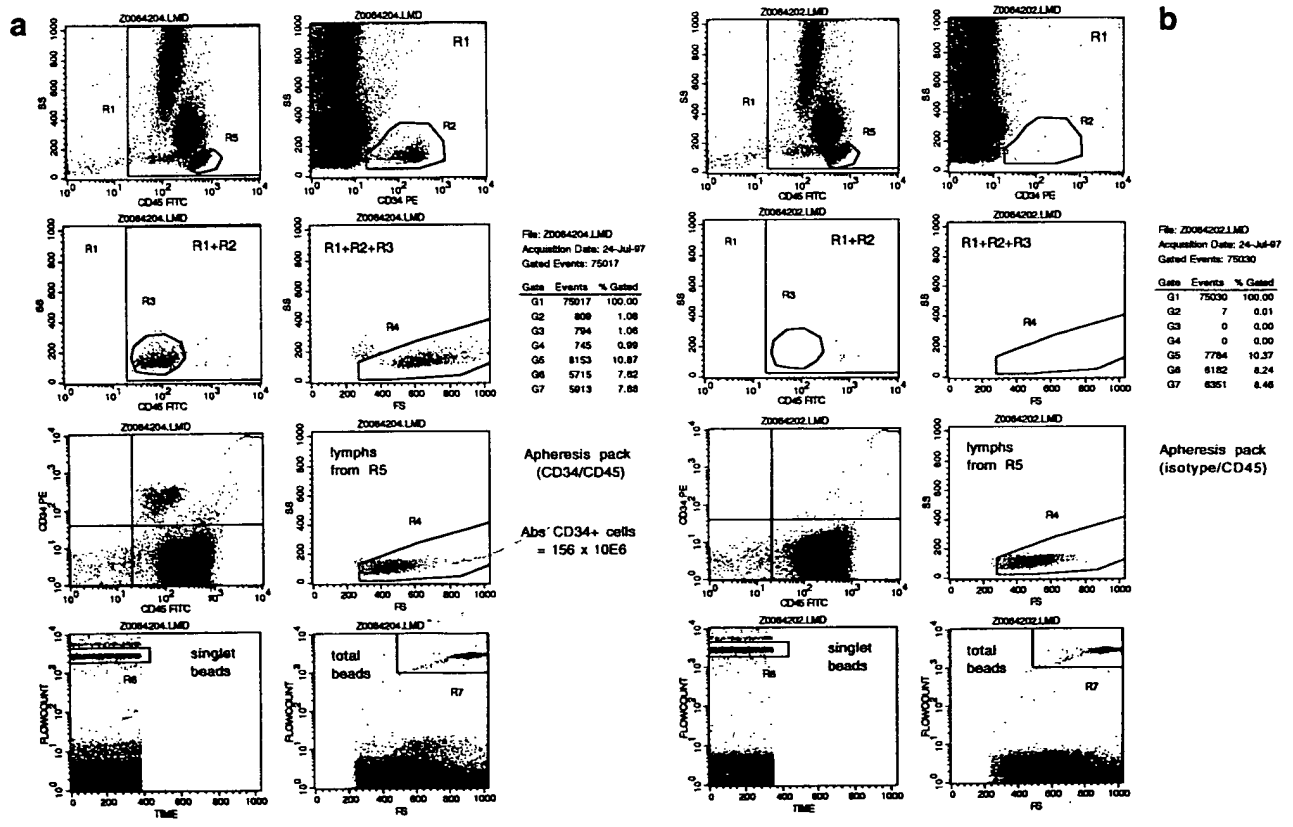

FIG. 2. Stem-kit gating strategy with (**a**) CD45 FITC/34 PE and (**b**) CD45 FITC/isoclonic control. Data collection and analysis were performed as described for Figure 1.

absolute number, the formula is as follows:

$$\text{Absolute Count (cells/}\mu\text{l)} = \frac{\text{Number of CD34+ cells} \times \text{Fluorospheres Concentration}}{\text{Number of Fluorospheres}}$$

The number of CD34 cells counted is R4. The number of fluorospheres counted is R6. The assayed Flow-Count Fluorosphere concentration is from the vial label. If the sample has been diluted before staining, the final answer must be multiplied by the dilution factor. To obtain the absolute CD34+ cell number per apheresis pack, the absolute count per microlitre obtained is multiplied by the dilution factor, the volume of the pack (in litres), and by 10^6 (to convert cells/μl to cells × 10^6/pack).

Cell Viability

In a separate experiment, the viability dye 7-AAD was added at a concentration of 1 μg/ml to a fresh and aged apheresis sample. A stock solution of 100 μg/ml in methanol was diluted 1:100 by adding 20 μL of the 7-AAD to the sample immediately after the addition of 2 ml ammonium chloride lysing solution. Samples were analysed after 15 min of incubation, with 7-AAD being detected in the fourth PMT of the Epics XL flow cytometer. This allows not only a direct assessment of absolute number of CD34+ cells but also discriminates between viable and nonviable CD34+ cells.

RESULTS

Linearity and Precision of Absolute Counting

Linearity of the single platform absolute counting method was determined by doubling dilutions of Stem-Trol KG1a control cells in Cyto-Trol lyophilised blood cells, over the range of 0–250/μl (Fig. 3). (Cyto-Trol was found to contain 2 CD34+ cells/μl, and this value was subtracted from the results obtained from the titration of Stem-Trol cells.) In these experiments, a minimum of 4,000 Flow-Count fluorospheres were collected on each duplicate analysis. The results of the duplicate analyses generated an R^2 value of 1.00.

Precision of the absolute counting method was measured at three target points (approximately 25, 125, and 200 CD34+ cells/μl) by replicate analysis of KG1a cells in normal whole blood. For each concentration of Stem-Trol KG1a cells, the sample was analysed eight times (Table 1). The coefficient of variation (C.V.) of replicate analysis varied from 4% to 9.9%.

Comparison of Single- Versus Dual-Platform Analysis

The correlation coefficient was 0.99, with the 72 paired patient samples indicating an excellent correlation between the two methods of analysis (Fig. 4). The Wilcoxon signed rank test showed that there was no statistically significant difference between the two methods ($P > 0.05$).

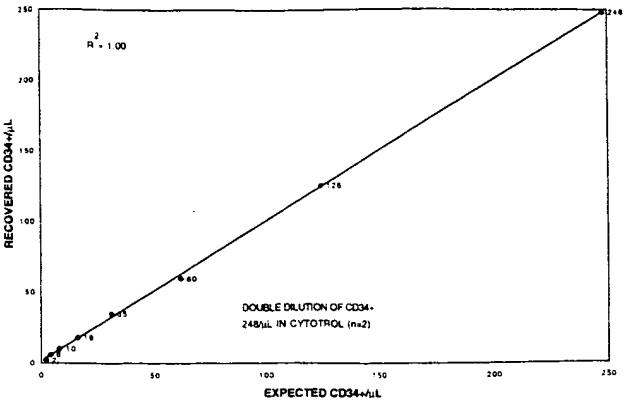

FIG. 3. Linearity of Stem-Trol control cells diluted with Cytro-Trol lyophilised cells over the range of 0–248/CD34+ cells/μl.

A Bland–Altman plot (Fig. 5) shows the majority of data points distributed randomly around the mean of the two methods. A few points fell outside the 2 S.D. range, particularly in the higher values.

Absolute Counting of Viable CD34+ Cells Using Single-Platform Methodology

Figure 6 shows the results of an experiment in which the absolute CD34+ cell count was determined on a fresh diluted apheresis sample and an aliquot of the same sample after 5 h on the laboratory bench. In this analysis, histogram 8 has been replaced by a 7-AAD versus side scatter analysis as detected in the fourth fluorescence PMT of the XL Flow Cytometer. The fresh apheresis pack contained a total of 352 × 10^6 CD34+ cells (Fig. 6a). The aged sample was analysed in the same manner except that the vital dye 7-AAD was added to separate viable cells from dead or dying cells. Analysis of this sample without taking into account the presence of the viability dye (Fig. 6b) revealed the same number of CD34+ cells as was determined on the fresh sample (Fig. 6a). However, as can be seen from the light scatter characteristics of the cells from the aged sample (R4), the single cluster of CD34+ cells identified in the fresh sample (Fig. 6a) had separated into two partly overlapping subsets in the aged sample (Fig. 6b). When the same listmode file was analysed by including in R7 only 7-AAD− (viable) cells (Fig. 6c), the absolute number of viable CD34+ cells was reduced by approximately 50% to 171 × 10^6. This cluster of viable cells retained the same light scatter characteristics as the viable cells in the original analysis (Fig. 6a). By moving R7 to include only 7-AAD+ cells (Fig. 6d), the remaining nonviable CD34+ cell population was found to be 181 × 10^6. These events formed a cluster characterised by lower FALS and slightly increased side scatter, typical of dead or dying CD34+ cells.

Figure 7 shows an example of the use of Flow-Count absolute counting beads with 7-AAD on an apheresis

Table 1
Precision Study

Expected	n	Minimum	Maximum	Mean	S.D.	C.V. (%)
25 CD34+/μL	8	22	28	25.4	2.5	9.9
125 CD34+/μL	8	110	139	125	10	8
212 CD34+/μL	8	180	203	193	8	4

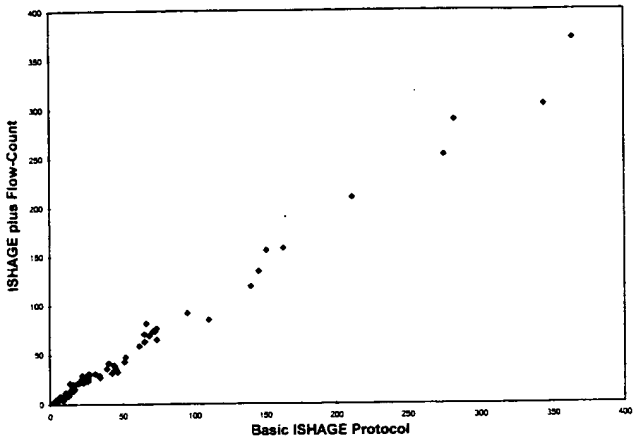

FIG. 4. Comparison of basic ISHAGE method to the ISHAGE with Flow-Count method.

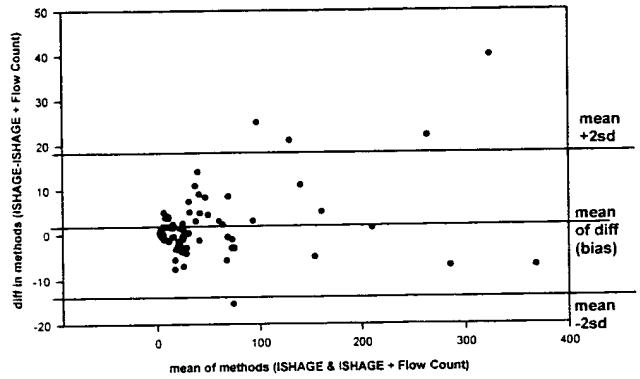

FIG. 5. Bland–Altman plot displaying differences of methods against their mean.

sample analysed on BD FACScan instrument. Histograms 1-6 were set as described for the Coulter XL instrument with CD45 and CD34 staining detected in channels FL1 and FL2, respectively. In contrast to the Coulter XL instrument, Flow-Count beads are detected on the FACScan at forward scatter levels lower than that exhibited by lymphocytes. Consequently, as shown in plot 7, the forward scatter threshold of the FACScan has to be lowered below that of lymphocytes, and the FL3 gain is lowered until the cluster of singlet beads (R6) can be distinguished on a FL3 versus forward scatter histogram. At these PMT settings, 7-AAD+ events are still easily detectable and can be gated (R7) for exclusion from further analysis. This is accomplished by setting logical gate (G1) = not R7 and R1. Histograms 5 and 6 are also logically gated as depicted to exclude the dead cells from R7.

DISCUSSION

The enumeration of CD34+ cells by flow cytometry is currently the most widely employed clinical method to evaluate the engraftment potential of peripheral blood stem cell collections. Clinical experience suggests that the reinfusion of approximately 2×10^6 CD34+ cells per kilogram body weight is generally sufficient to produce prompt and sustained engraftment (9). Despite the proliferation of flow cytometric methods (10–20), analysis of rare events such as stem cells presents a challenge to the flow cytometry laboratory [reviewed in (26)]. Several interlaboratory surveys undertaken in different continents have described an alarming lack of reproducibility for a given CD34 result (16–18). A reduction in the number of variables present in the test is required if inter- and intralaboratory comparisons are to achieve greater concordance.

Until recently, absolute CD34+ cell counts were generally derived from so-called two-platform analysis in which the percentage of CD34+ cells are first enumerated by flow cytometry against a denominator of total nucleated cells (10,12,14,15) or total CD45+ cells (13,19,20). This percentage of CD34+ cells is then multiplied by the LKC derived from an automated hematology analyser to generate the absolute CD34+ cell count. So-called single-platform methods reduce variability by eliminating the need for the cell count from an automated analyzer.

By mixing a known amount of fluorescent microbeads with a stained blood sample, the absolute cell count can be derived directly from the flow cytometer by measuring the ratio of beads and the CD34+ cells in a sample. Absolute count single-platform methods have been described for CD4/CD8 enumeration (27,28) and CD34 (29,30). In the present study, we modified the basic two-colour ISHAGE protocol (19) by adding Flow-Count microspheres to generate an absolute count. Comparison of the basic ISHAGE protocol with the single-platform ISHAGE protocol showed a high degree of concordance over a wide range of CD34 counts (correlation coefficient $r = 0.99$). A Bland-Altmann plot did not reveal significant bias. Linearity and precision of the absolute counting method was also confirmed over a range of target CD34 values.

This modification of the basic ISHAGE protocol combines the accuracy and sensitivity provided by the sequential gating strategy at the heart of the original method with the simplicity of absolute counting on a single-instrument platform. A similar approach is used in the ProCOUNT kit by Becton Dickinson (29,30), which uses CD34 and CD45 antibody conjugates alongside a nucleic acid dye to separate nucleated leukocytes from other events and debris in

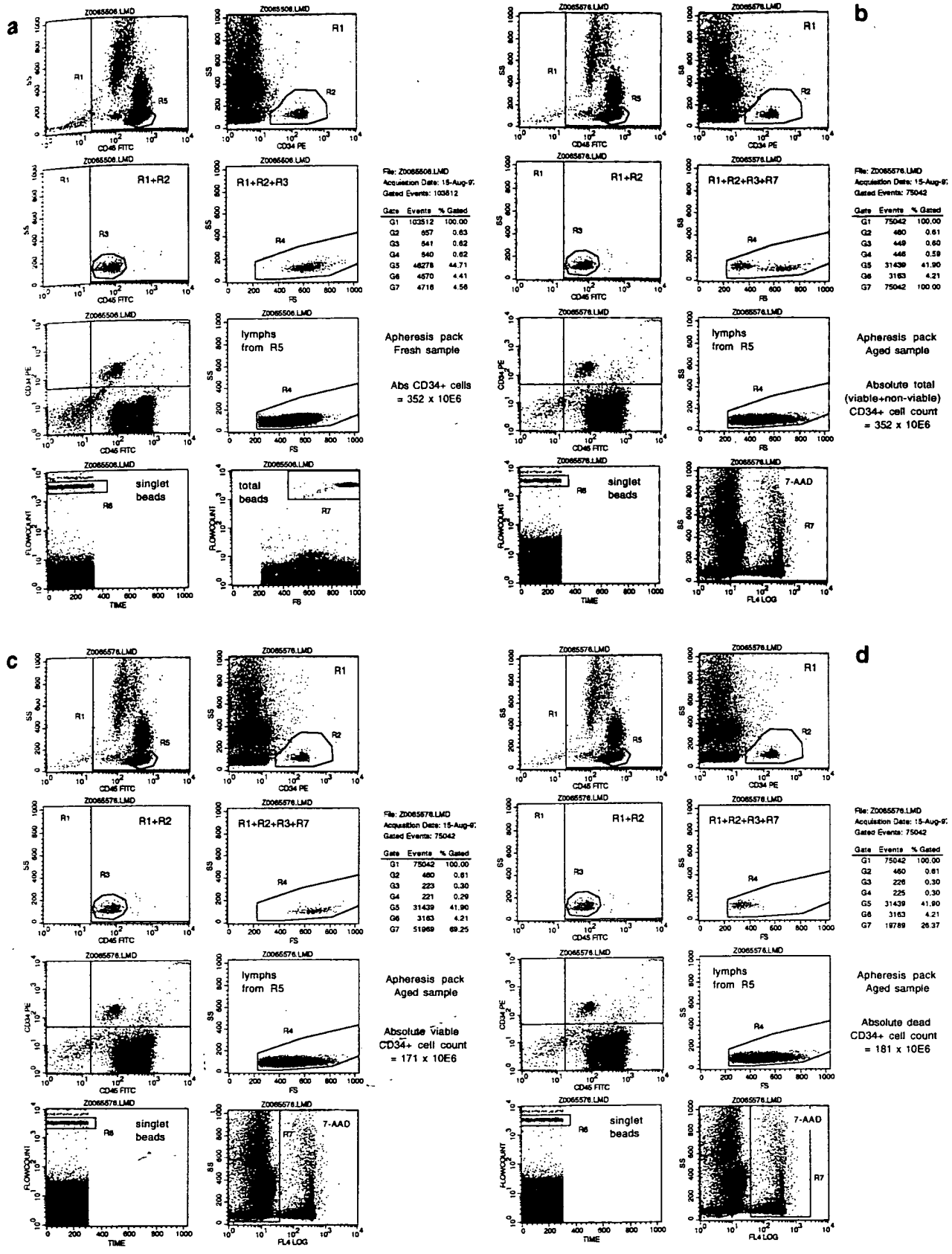

FIG. 6. Stem-Kit with 7-AAD. **a:** Fresh apheresis pack. **b:** At 5 h gated on all cells. **c:** At 5 h gated on live cells. **d:** At 5 h gated on dead cells.

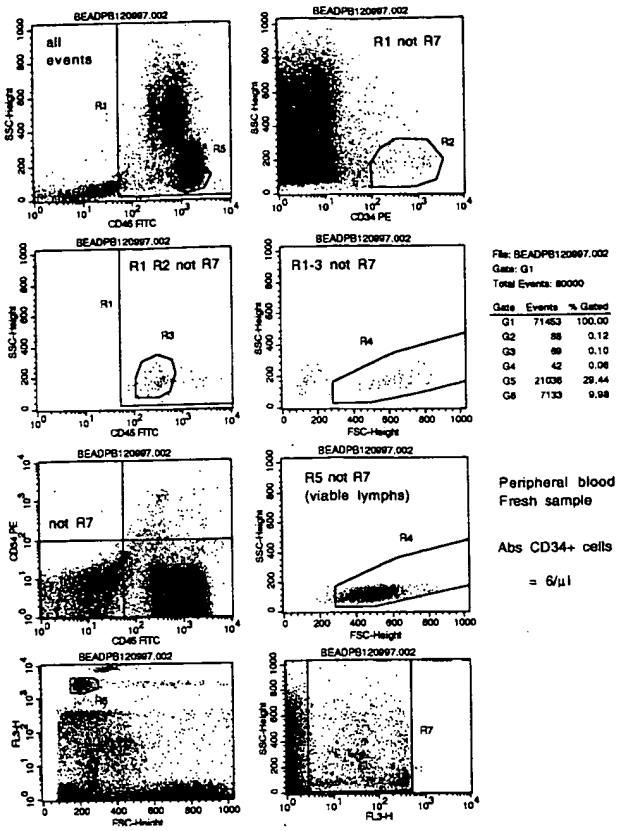

FIG. 7. Stem-Kit and 7-AAD on a fresh peripheral blood sample run on a BD FACScan. The threshold setting on forward scatter (histogram 7, bottom left) is set below that of the lymphocyte population to ensure beads are not excluded from analysis.

a lyse-no-wash protocol. In contrast to the ProCOUNT kit, however, this modified ISHAGE protocol retains the flexibility of our original method because the addition of the bead requires only two of the three (or four) fluorescence channels of a typical clinical flow cytometer, as the beads can be detected and enumerated in any fluorescence channel. The remaining fluorescence detectors are thus available for other analytical purposes such as qualitative analysis of the CD34+ cell fraction using additional antibody conjugate(s) or the inclusion of live:dead cell discriminator dyes (31,32). Because the present study was conducted on a Coulter XL with four fluorescence detectors, beads were displayed as a separate histogram on FL3 for convenience.

The feasibility of such an approach is illustrated by the addition of 7-AAD, a G-C base-pair specific DNA intercalator, as a dye for detection of nonviable cells. By combining the modified single-platform ISHAGE method with 7-AAD, we were able to detect the absolute number of viable and nonviable CD34+ cells. The ability to distinguish dead non-CD34+ cells that have been nonspecifically stained and dead CD34+ cells from viable, bona fide CD34+ cells is of potentially great clinical importance. Although the cumulative gating strategy used in the ISHAGE method is highly efficient at excluding nonspecifically stained non-CD34+ cells from the analysis (26,32,33), detection of nonviable CD34+ cells presents a more formidable problem. Schmid et al. (21) were the first to describe the use of 7-AAD as a dye for detection of nonviable cells by demonstrating that different subsets of thymocytes undergoing apoptosis could be visualised by using this dye and multivariate analysis. Owens and Loken (14) described the use of 7-AAD in CD34 analysis as a means of excluding nonviable cells from subsequent CD34 analysis. However, they did not extend this to the use of the viability dye to look at viable versus nonviable CD34+ cells. Our modified methodology has a number of clinical applications including an ability to assess the viability of selected CD34+ cell fractions after purging (to remove for example residual tumor cells) or the analysis of postcryopreservation samples, procedures that may adversely affect the yield and viability of CD34+ fraction.

The original ISHAGE protocol could be performed on a variety of flow cytometers. As described in detail above, minor modifications to the FACScan instrument settings are required, and the logical gate definitions are established to exclude the 7-AAD+ events from further analysis. Although not formally tested herein, it should also be possible to perform similar analysis on the dual-laser BD FACSCALIBUR instrument and a variety of commercially available cell sorters.

Other benefits of including an internal reference bead in single-platform methods in a three- (or four-) color analysis is that the CD45 positivity is no longer used as a denominator in the calculation of absolute CD34+ cells. Thus, controversial issues such as "what is the true denominator," nucleated LKCs (CD45+ events), or total nucleated cells (DNA dye + events) (29) can be avoided. Instead, the CD45 expression is used solely as part of the sequential gating strategy to identify accurately bona fide CD34+ cells. Moreover, the operator does not need to be concerned about correction for the presence of nucleated red blood cells (that express little or no CD34 and CD45) and platelet aggregates that may be counted as "leukocytes" by some automated hematology analysers currently in use.

It is interesting to note that, in the present study and those previously cited (27,28), although excellent correlation has been found, there are differences between some individual samples. Accurate dilution of the blood or apheresis pack is essential in any flow-cytometry-based absolute counting method because the final result is multiplied by this dilution factor. In addition, when examining apheresis packs, the LKC may vary significantly, requiring a haematology analyser to determine the initial count before dilution. Failure to dilute the sample into an appropriate range ($<20 \times 10^9$/l) may result in poor sample staining due to nonsaturating conditions for the monoclonal antibodies. Precise pipetting of sample and fluorospheres is also essential because the absolute count is dependent on an accurate measurement of CD34+ cells and fluorospheres. In the present study, a positive displacement pipette was used for both patient sample and beads. Nicholson et al. (28), by using TruCount tubes (Becton Dickinson) that contain a premeasured number of lyophi-

lised beads to determine lymphocyte subsets, found good reproducibility with the single-platform method, with results 5–10% higher than those obtained by the conventional two-platform method, suggesting a potential bias. The use of a Bland–Altman-type bias plot is strongly recommended when analysing these types of data because correlation and regression analysis do not provide sufficient resolution (22).

The inclusion of a stabilised suspension of the Stem-Trol control KG1a cell line in the Stem-Kit at a known concentration is useful in determining and monitoring the accuracy of the pipetting steps of this method. In addition, because this control material can be diluted in peripheral blood, it can also be used as a "process control" undergoing staining and lysis exactly as performed on the test samples. Because the stabilised KG1a cells have been engineered to express CD34 and CD45 antigens at levels comparable to antigens of normal CD34+ cells (25), their staining pattern with respect to CD34/CD45 reagents can be used as an extra internal control to ensure that the FL1, FL2, and light scatter parameters of the cytometer are adequately set.

The use of appropriate fluorochromes and monoclonal antibodies is also very important in protocols using lyse-no-wash sample preparation. Regarding the CD45-FITC reagent, stained lymphocytes should at least reach the third decade of the four-decade log amplifier. At the appropriate amplifier gain setting, there is excellent discrimination between true CD45+ and CD45− events when using this reagent. Other conjugates may not give this level of resolution (unpublished observations). As can be seen in Figures 1, 2, and 6, there is a two-decade log shift between CD45− events and the lymphocyte cluster, allowing clear discrimination of a cluster of true CD34+ cells that express only dim levels of CD45. As detailed in the ISHAGE guidelines (19) and elsewhere (26,34), CD34 antibodies detecting class III epitopes (4) appear to be the most reliable reagents for routine CD34+ cell determinations in both normal and abnormal samples by flow cytometry.

Samples processed for two-platform methodologies generally include washing/centrifugation steps that can lead to the differential loss of specific leukocyte subsets (35). Some red cell lysing reagents contain fixatives that affect the fluorescence intensity of cells stained with fluorochrome-labelled monoclonal antibodies (12,36). Furthermore, different lysing reagents can have unpredictable effects on the light scatter properties of cells. The use of an ammonium chloride lyse-no-wash sample preparation eliminates the potential loss of cells and/or beads through washing/centrifugation. Moreover, the use of ammonium chloride neither unduly affects the light scatter characteristics of the cells nor appears to alter the stability of antibody:antigen interactions if samples are analyzed within 1 h of preparation.

Stem-Kit does not use an isotype control but rather a mixture of conjugated and unlabelled CD34 antibody to enumerate nonspecifically stained events. However, given the selectivity of the sequential gating strategy used in this protocol, which takes into account both positivity and intensity of antigen expression, it is likely that the isoclonic control may also be redundant. In this set of data, only three samples exhibited background staining with the isoclonic control above 1/μl.

In summary, adding a known number of fluorescent beads to the ISHAGE protocol allows the determination of absolute CD34 counts without the added variable of a haematology analyser. Comparison of the basic ISHAGE protocol with the Stem-Kit absolute counting modification showed excellent correlation and agreement. The latter is rapid (processing time is approximately 35 min, including acquisition and analysis), simple, and highly accurate, thereby making it suitable for the clinical laboratory. This modification combines the flexibility of the original ISHAGE method, including the ability to add a viability dye to the analysis of autografts that have been purged, frozen, or otherwise manipulated, a feature not possible in other currently available absolute counting methods. The interinstitutional reproducibility of CD34 results by flow cytometric methods will require standardised instrument set-up calibration and protocols in addition to standardized analysis of samples. In this regard, the efforts and experience of our European colleagues related to instrument standardization (37) and workshop approach to sample preparation and analysis (15) should not be overlooked.

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Thanks a bunch!!!

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Isolation of neutrophil precursors from bone marrow for biochemical and transcriptional analysis

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Abstract

The neutrophilic granulocyte is the most numerous leukocyte in peripheral blood. The development from a multipotent progenitor cell to a mature neutrophil takes place in the bone marrow over a period of 10–14 days. In order to understand the cellular mechanisms behind this process, it is necessary to investigate cells from different stages of neutrophil differentiation. As no human cell line has the ability to faithfully reproduce the entire differentiation process from promyelocyte to segmented neutrophil the analysis of many maturation-dependent processes has to be done on neutrophil precursors from human bone marrow. For this purpose, a technique whereby neutrophil precursors can be isolated from the bone marrow and separated according to their maturity is required. Two different methods have been shown to be useful for isolation of immature neutrophils: density centrifugation on a Percoll gradient, where the increasing density of the cells with maturity forms the basis of the separation, and multidimensional flow cytometry, where a combination of size, granulation, and surface markers are used for the discrimination of different neutrophil precursors. This paper will review these two methods for separation of neutrophil precursors with special emphasis on Percoll density centrifugation and the use of cells isolated by this technique for the analysis of neutrophil-specific mRNAs and the biosynthesis of neutrophil granule proteins.

Keywords: Myelopoiesis; Percoll density centrifugation; Biosynthesis; Gene transcription

1. Introduction

The neutrophilic granulocyte is the most numerous leukocyte in peripheral blood. The neutrophils are part of the first line of defense against invading microorganisms and are unique among cells by possessing four types of exocytosable storage organelles (azurophil-, specific-, and gelatinase granula and secretory vesicles) (Borregaard and Cowland, 1997). Each of these exocytosable organelles is characterized by its specific content of enzymes and antimicrobial proteins which are used by the neutrophil to exert its different biological functions (Borregaard and Cowland, 1997). The three granule types and the secretory vesicles are formed sequentially during granulopoiesis in the bone marrow and their appearance can be used to mark the stage of neutrophil differentiation (Borregaard and Cowland, 1997). The maturation of the neutrophil is also characterized by a gradual condensation of the nucleus which, in combination with the cellular granulation, results in distinct morphologies of the different neutrophil precursors. These maturation-associated changes of the

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morphology are accompanied by the sequential appearance of transcripts for granule and membrane proteins and a concomitant synthesis of their cognate proteins (Borregaard and Cowland, 1997; Cowland and Borregaard, 1999).

The study of granulocytic cell differentiation is important as it allows one to investigate (a) the regulation of maturation-associated gene expression and protein synthesis, (b) the intracellular targeting and sorting mechanisms that enables the neutrophil to specifically direct and store its different granule proteins into only one or a few of the four exocytosable organelles, and (c) the cellular mechanisms that are corrupted in those forms of acute myeloid leukemia (AMLs) that are characterized by the accumulation of immature neutrophils.

No human myeloid cell line can faithfully reproduce the entire process of neutrophil maturation. Although cell lines such as HL-60 (Breitmann et al., 1980) and NB4 (Lanotte et al., 1991) can be induced by retinoic acid to form morphologically mature PMNs, they lack specific- and gelatinase granula (Johnston et al., 1992; Khanna Gupta et al., 1994; Khanna Gupta et al., 1996) and thus cannot be used to outline the terminal differentiation of neutrophils. For such purposes, neutrophil precursors from human bone marrow are required. This review describes different methods for isolation of neutrophil precursors from bone marrow and the use of these cells for the study of mRNA and protein synthesis.

2. Isolation of neutrophil precursors from bone marrow by density centrifugation

2.1. Separation of blood cells by density centrifugation

Preparation of leukocytes from peripheral blood by Ficoll-Isopaque (e.g., Lymphoprep™, Nycomed Amersham) density centrifugation is a technique employed by many laboratories working with mononuclear cells and granulocytes. This method relies on the differences in buoyant density between mononuclear leukocytes (monocytes and lymphocytes have a density < 1.077 g/ml) and granulocytes (which have a density > 1.077 g/ml) (Olofsson et al., 1980). In the density gradient, the cells will sediment to the position where the density of the surrounding media is equal to the density of the cells. In the case where a separation media with a fixed density is used, this media will function as a density barrier. This means that cells with a density below that of the separation media will be stopped at the interface between the cell suspension and the separation media while cells with a higher density will sediment through the media and can be recovered as a pellet. As Ficoll-Isopaque has a density of 1.077, centrifugation of peripheral blood cells in this media will result in a band at the border between the cell solution and the separation media containing the mononuclear cells and a pellet containing neutrophils and erythrocytes (Böyum, 1968).

Maturation of granulocytes is accompanied by a progressive increase in the density of the neutrophil precursors (Olofsson et al., 1980). This is probably a direct consequence of the increased granulation of the cells (Winqvist et al., 1982; Sitar and Fornasari, 1989), a notion that is strengthened by observing that day-6 retinoic-acid-induced HL-60 cells, which resemble fully mature PMNs except for their lack of specific- and gelatinase granula (Khanna Gupta et al., 1996) sediment in a Percoll gradient at a density < 1.072 g/ml (Martin et al., 1990) in contrast to PMNs from blood which have a buoyant density of > 1.080 g/ml (Olofsson et al., 1980). This relationship between the maturity and density of the neutrophil precursors means that different populations of neutrophil precursors can be isolated by density centrifugation in a manner analogous to the methods used for isolation of leukocyte subgroups from peripheral blood.

Although media such as albumin has been used for density separation of bone marrow cells (Shortman, 1968; Moore et al., 1973), Percoll® (Amersham-Pharmacia Biotech, Uppsala, Sweden) has been found to be the gradient medium of choice due to its ease of handling and the reproducibility of the separation profiles. Percoll contains polyvinylpyrrolidone coated silica beads and is very suitable for density centrifugation of cells as the medium is non-toxic, can be adjusted to physiological ionic strength and pH, and is iso-osmotic throughout the gradient (Pertoft et al., 1978). Both continuous (Uyesaka et al., 1989) and discontinuous two-layer (Borregaard et al., 1995) Percoll gradients

have been used for isolation of neutrophil precursors from human bone marrow.

2.2. Isolation of neutrophil precursors from bone marrow on a continuous Percoll gradient

Separation of white blood cells from human bone marrow on a continuous Percoll gradient (density range: 1.030–1.110) and subsequent collection of 0.5-ml fractions from the bottom of the tube could resolve neutrophil precursors into five populations (Olofsson et al., 1980): myeloblasts (peak concentration (p.c.) at the density 1.064 g/ml), promyelocytes (these cells had a bimodal distribution with peak levels at 1.066 and 1.076 g/ml), myelocytes (p.c. at 1.076 g/ml), metamyelocytes (p.c. at 1.079 g/ml), and mature neutrophils (p.c. at 1.086 g/ml). However, a considerable overlap between the five cell populations was observed due to heterogeneity in the density of the different neutrophil precursors. For this reason, the highest purity obtained in a single fraction was 20.8% for myeloblasts, 14.2% for promyelocytes, 40.0% for myelocytes, 27.3% for metamyelocytes, and 87.7% for mature granulocytes. Although these data clearly demonstrate that neutrophil precursors can be separated on the basis of density, the considerable overlap between the five cell populations and the fact that an 11-layer Percoll gradient had to be made in order to form the continuous gradient after centrifugation (Olofsson et al., 1980) renders this technique somewhat difficult to use in practice. To our knowledge this separation technique has not been used for the isolation of immature neutrophils for biochemical studies.

2.3. Isolation of neutrophil precursors from bone marrow on a two-step discontinuous Percoll gradient

Separation of neutrophil precursors can also be achieved on a discontinuous Percoll gradient. By using two separation media of different densities, two density barriers are introduced (one between the cell solution and the light Percoll medium and one between the light and heavy Percoll media). We have used this technique to fractionate bone marrow derived cells of the neutrophil lineage into three cell populations consisting of early immature, late immature, and mature neutrophils, respectively (Borregaard et al., 1995). This method, which is very reproducible and easy to handle, has been used with success for both immunocytochemical staining (Borregaard et al., 1995; Sørensen et al., 1997; Volck et al., 1998) biosynthesis studies (Borregaard et al., 1995; Sørensen et al., 1997; Arnljots et al., 1998), and transcriptional analysis of human neutrophil precursors (Cowland and Borregaard, 1999). The same separation protocol has also been used for isolation of neutrophil precursors from rabbit bone marrow (Zarember et al., 1997). The method is described in detail below.

2.3.1. Isolation of leukocytes from bone marrow

Bone marrow cells were obtained by aspiration from the posterior superior iliac crest of healthy volunteers. The 20-ml syringe used for aspiration was preloaded with 5 ml sterile ACD (25 mM sodium citrate, 126 mM glucose) and 15 ml bone marrow was aspirated into the syringe. The cell suspension was transferred to a 50-ml Falcon® tube (Becton Dickinson, NJ, USA) and an equal volume of 2% Dextran T-500 (Amersham–Pharmacia Biotech) in 0.9% NaCl was added to induce sedimentation of erythrocytes. Following sedimentation (15–20 min), the clear supernatant containing leukocytes was removed and the cells recovered by centrifugation (200 × *g* for 10 min at room temperature). The pellet was resuspended in 54 ml PBS (4°C) which was added slowly while vortexing the tube at medium speed. The cells were kept on ice until used.

2.3.2. Preparation of the Percoll gradient

Six 50-ml Falcon tubes were required for the density centrifugation. Each tube was filled with 9 ml Percoll media with a density of 1.065 g/ml (for preparation of Percoll media of different densities — see below). This media was carefully underlayered with 9 ml Percoll media with a density of 1.080 g/ml by the use of a syringe with a 14-G needle attached — a sharp line between the two Percoll media must be visible for the gradient to be usable. The tubes were kept on ice until used.

2.3.3. Percoll centrifugation

Nine milliliters of bone marrow leukocytes suspended in PBS was layered very gently on top of

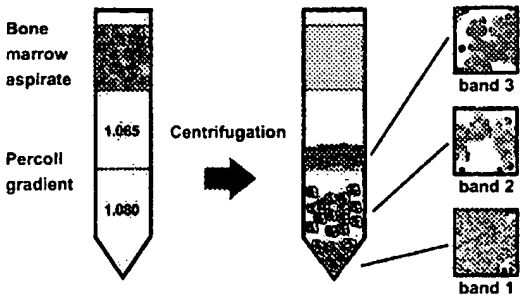

Fig. 1. Schematic drawing of the principle of Percoll centrifugation for the separation of neutrophil precursor from human bone marrow. The bone marrow aspirate is placed upon a two-layer Percoll gradient of densities 1.065 and 1.080 g/ml. Following centrifugation, three cell bands can be recovered: "band 1" containing band- and segmented neutrophil, "band 2" containing myelocytes and metamyelocytes, and "band 3" containing myeloblasts and promyelocytes.

each of the six two-layer Percoll gradients and the tubes were centrifuged (1000 × *g* for 20 min at 4°C). This resulted in a separation of the bone marrow cells into three "bands" (Fig. 1): "band 1" — the pellet at the bottom of the tube — which contained the most mature neutrophils (primarily band and segmented neutrophils) as well as some contaminating erythrocytes; "band 2" — the cells floating in the lower Percoll medium just above the pellet — which contained the late immature cells (primarily myelocytes and metamyelocytes); and "band 3" — the cells found at the interface between the two Percoll media — which contained the early immature cells (primarily myeloblasts and promyelocytes). Table 1 shows a representative differential count of the neutrophil precursors in the three bands. Some co-purification of cells that do not belong to the neutrophil lineage also occurs, as noted in Table 1. This should be taken into consideration if very pure neutrophil cell populations are required for the experiments.

2.3.4. Retrieval of the neutrophil precursors

Following centrifugation, the three "bands" as well as a layer consisting of lipids and cellular debris above the upper (light) Percoll medium, could be seen. The "band 3" cells were found at the 10-ml marking — these cells were removed with a serum pipette. Between 5 and 7 ml was removed from each tube and the cells from the two tubes were pooled in a new 50-ml Falcon tube. This resulted in three tubes with "band 3" cells which were stored on ice. Next, the "band 2" cells, which form a fuzzy layer just above the cell pellet, were removed with a serum pipette in a volume of 4–5 ml from each gradient. These cells were pooled and stored as above. Finally, the remaining Percoll and PBS was removed by vacuum-suction leaving only the pellet containing the "band 1" cells. The pellets were resuspended in distilled water by gentle vortexing in order to remove contaminating erythrocytes by hypotonic lysis: first, the pellet of one tube was resuspended in 10 ml distilled water, then the suspension was transferred to a second tube and the pellet in this tube resuspended, and then, after 25–30 s, 10 ml 1.8% NaCl was added to obtain a final NaCl concentration of 0.9%. The three tubes of "band 1" cells were stored on ice until used.

In order to remove the remaining Percoll, all tubes were filled to 50 ml with cold (4°C) 0.9% NaCl and centrifuged (200 × *g* for 10 min at 4°C). The pellets of the "band 1", "band 2", and "band 3" cells, respectively, were resuspended in a total volume of 10 ml cold (4°C) 0.9% NaCl and pooled. The number and viability of the cells were deter-

Table 1
Differential count of myeloid cells
The distribution of neutrophil precursors in the three bands of Percoll density gradient separated bone marrow cells. The numbers refer to the percentage of the different cell types in each band based on a differential count of 200 neutrophil precursors from a representative Percoll separation. Some co-purification of erythroblasts (1.5%, 10.5%, and 23.5% of the total cell population in bands 1, 2, and 3 cells, respectively) and eosinophils (8.5% of total band 1 cells) was observed.

	Segmented	Bands	Metamyelocytes	Myelocytes	Promyelocytes	Mycloblasts
Band 1	36.5	52.5	9.5	1.0	0.0	0.5
Band 2	0.5	19.0	42.5	33.5	4.0	0.5
Band 3	0.5	3.5	4.5	12.5	73.0	6.0

mined by Trypan-blue staining and the distribution of neutrophil precursors in the three bands was assessed by differential counting of cytospins (2×10^5 cells). The cells were now ready for further studies.

2.3.5. Preparation of the Percoll density medium

The Percoll stock had to be diluted to the correct density in sterile water and 1/10th of the final volume of 10 × PBS (10 × PBS is 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7\ H_2O$, 1.4 mM KH_2PO_4 (pH 7.4)) before being used for separation of bone marrow cells. The pH of the Percoll solutions were adjusted to 7.4 before use. The general formula for calculating the amount of Percoll stock required for making the working solution is:

$$V_P = V_f(\rho_{Pf} - 0.1\rho_S - 0.9)/(\rho_{Ps} - 1)$$

where V_P = volume of Percoll stock used in milliliters, V_f = final working solution in milliliters, ρ_{Pf} = final density of Percoll in media, ρ_S = density of the 10 × salt (buffer) solution, and ρ_{Ps} = density of Percoll in the stock solution. As 10 × PBS has a density of 1.056, the formula can be rewritten to:

$$V_P = V_f(\rho_{Pf} - 1.0056)/(\rho_{Ps} - 1)$$

when the Percoll gradient is used for separation of bone marrow neutrophils.

3. Isolation of bone marrow cells by use of antibodies

At least two different antibody-based approaches have been used to isolate blood cells: by antibodies coupled to magnetic beads and isolation of the labeled cells with a strong magnet and by fluorescent-labeled antibodies followed by collection of the labeled cells by flow cytometry and cell-sorting.

3.1. Isolation of blood cells with magnetic immunobeads

Isolation of very pure populations of leukocyte subgroups from peripheral blood with antibodies against lineage restricted antigens (e.g. CD19 for B-lymphoid cells) has been achieved with antibody-coated magnetic beads (Funderud et al., 1990). In the same manner, highly purified $CD34^+$ progenitor cells have been isolated from human bone marrow (de Coutinho et al., 1995; Servida et al., 1996). This method can, however, not by itself be used for isolation of neutrophil precursors at different stages of maturity due to the lack of lineage restricted or unique combinations of surface markers for these cells (Terstappen et al., 1990).

3.2. Isolation of neutrophil precursors from bone marrow by flow cytometry

By combining the size and granularity of bone marrow cells (measured as forward and orthogonal light scattering, respectively) with the measurement of the neutrophil-associated antigens CD11b, CD15, and CD16 (recognized by antibodies labeled with three different fluorescent dyes), it is possible to sort neutrophil precursors according to maturity by multidimensional flow cytometry (Terstappen et al., 1990). By this technique, the neutrophil precursors can be sorted into six classes containing cells of increasing maturity: N-I (myeloblasts), N-II (promyelocytes and early myelocytes), N-III (predominantly early myelocytes), N-IV (myelocytes), N-V (predominantly metamyelocytes and band cells), and N-VI (metamyelocytes, band cells, and segmented neutrophils). Although it is possible with this technique to obtain very pure cell populations for immunocytochemical analysis, it is difficult, due to the limited number of cells that can be sorted in one event, to obtain enough cells for biochemical and transcriptional analysis. Another problem is that most flow cytometers cannot collect six different cell populations in one sorting event. This means that part of the bone marrow sample must be run more than once or that multiple bone marrow samples have to be sorted.

Multidimensional flow cytometry has been used with success for the diagnosis of acute myeloid leukemia and for follow-up studies after treatment of the leukemic patients (Terstappen et al., 1992; Porwit et al., 1996). The method has, however, not been used for the analysis of protein biosynthesis or gene transcription in neutrophil precursors — probably due to the limitations described above. Recently, a method for measuring the levels of gene expression in in vitro differentiated $CD34^+$,$CD38^-$ cells at the single-cell level was described (Cheng et al., 1996). The $CD34^+$,$CD38^-$ cells were isolated from bone marrow by cell sorting and the transcript levels were measured by a combination of reverse transcription–

PCR and Southern blotting. A similar approach can probably be used for measuring mRNA levels in neutrophil precursors isolated by multidimensional flow cytometry.

4. Pros and cons of Percoll vs. antibody-mediated isolation of blood cells

No comparative studies of the possible effects of purifying neutrophil precursors by Percoll density centrifugation contra antibody-based methods have been published. The use of magnetic beads coated with antibodies against CD15 for isolation of neutrophils from peripheral blood has been shown to result in a more pure population of neutrophil granulocytes (> 99%) than that obtained by Percoll density centrifugation where the neutrophils were contaminated with 6% eosinophils (Zahler et al., 1997). The neutrophils isolated by magnetic separation also appeared to be less activated than those purified on the Percoll gradient as they had a lower basal surface expression of CD11b and demonstrated a more pronounced upregulation of CD11b in response to fMLP. In another study, where removal of neutrophils with anti-CD16-coated magnetic beads was included as part of the purification of eosinophils, it was found that the isolation procedure affected the chemotactic responsiveness of the eosinophils to IL-8 (Rozell et al., 1996). In contrast, eosinophils isolated by Percoll density centrifugation were still able to respond to IL-8. Why magnetic separation had this effect on the eosinophils is not clear. It is, however, known that the use of antibodies for isolation of blood cells in some cases can result in an activation of the cells. This has been observed for e.g. T-cells incubated with anti-CD3 and anti-CD28 (Levine et al., 1996) and neutrophils incubated with anti-CD11b and anti-CD16 (Bartunkova et al., 1997). The use and choice of antibodies for cell purification must therefore be considered carefully.

5. Analysis of Percoll-separated bone marrow cells

We have used Percoll-separated bone marrow cells to investigate the regulation and synthesis of granule proteins in neutrophil precursors. A short description of the some of the techniques employed, and the results obtained, is presented below.

5.1. Identification of granule proteins in neutrophil precursors by immunocytochemical staining

Cytospin preparations of neutrophil precursors isolated by density centrifugation have been analyzed for the presence of the granule proteins myeloperoxidase (MPO), lactoferrin, hCAP-18, YKL-40, and gelatinase (Borregaard et al., 1995; Sørensen et al., 1997; Volck et al., 1998). In short, cytospins of the neutrophil precursors were fixed in 4% formaldehyde, 0.1 M phosphate buffer (pH 7.0) and permiabilized in TBS (50 mM Tris–HCl (pH 7.6), 150 mM NaCl) with 1% Triton X-100. Unspecific binding was blocked by incubating with TBS containing 1% bovine serum albumin (BSA) and the cells were incubated with the "anti-granula protein"-antibody diluted in TBS, 0.25% BSA. Following wash in TBS the immunoreaction was developed by alkaline phosphatase–antialkaline phosphatase (APAAP) staining (Dako, Glostrup, Denmark). The slides were counterstained in Mayer's hematoxylin.

Positive staining for MPO was seen in cells from all three bands in accordance with its synthesis in promyelocytes and storage in azurophil granules (Borregaard and Cowland, 1997). Positive staining for lactoferrin (Borregaard et al., 1995), YKL-40 (Volck et al., 1998), and hCAP-18 (Sørensen et al., 1997) was found only in "band 2" and "band 1" cells as expected for proteins synthesized in myelocytes/metamyelocytes and stored in specific granules (Borregaard and Cowland, 1997). Finally, positive staining of gelatinase was observed only in band 1 cells indicating that the synthesis of gelatinase and the formation of gelatinase granula occurs in band/segmented cells (Borregaard et al., 1995). Immunocytochemical staining of band 2 cells (myelocytes/metamyelocytes) with anti-MPO and anti-lactoferrin antibodies is shown in Fig. 2. All the cells were found to stain positive for the azurophil granule protein MPO whereas only the most mature fraction of the cells showed a strong staining for lactoferrin.

5.2. Biosynthesis of granule proteins

The biosynthesis of the granule proteins MPO, elastase, defensin, lactoferrin, NGAL, hCAP-18, gelatinase, and lysozyme (Borregaard et al., 1995;

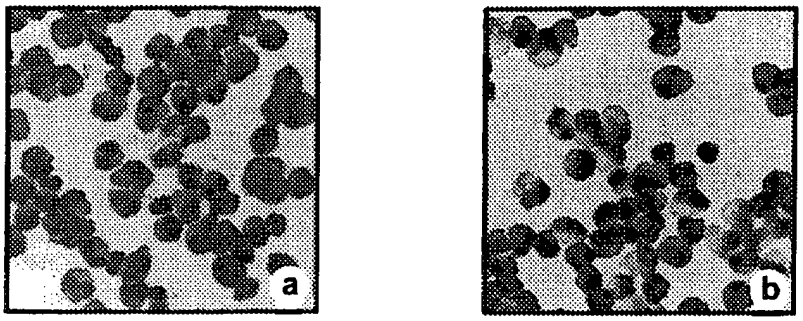

Fig. 2. Immunocytochemical staining of bone marrow cells. Cytospin preparations of cells from band 2 were labeled with antibodies against MPO (a) and lactoferrin (b).

Sørensen et al., 1997; Arnljots et al., 1998) has been investigated in the three Percoll-isolated cell populations. There are slight differences in the protocols depending on which protein is analyzed but typically the cells from the three bands were resuspended to 1×10^7 cells/ml in methionine-free MEM (minimum essential medium — with Earle's salt (Gibco BRL, Life Technologies, Paisley, Scotland)) supplemented with 10% fetal calf serum (FCS) and incubated for 45 min at 37°C. The cells were pelleted and resuspended to 3×10^7 cells/ml in the above medium supplemented with 200 μCi/ml [^{35}S]-methionine (ICN Pharmaceuticals, Plainview, NY) and incubated for 30 min at 37°C. The pulse was stopped by pelleting the cells and washing once in PBS. The cells were resuspended to 1×10^7 cells/ml in RPMI-1640 (Gibco BRL) with 10% FCS. After 2 h incubation the cells were pelleted and resuspended to 3×10^7 cells/ml in RIPA buffer (150 mM NaCl, 30 mM HEPES (pH 7.3), 1% (vol/vol) Triton X-100, 1% (wt/vol) sodium deoxycholate, 1% (wt/vol) sodium dodecyl sulfate (SDS)) containing protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 200 U/ml aprotinin, 100 μg/ml leupeptin) and incubated overnight at 4°C. Undissolved material was pelleted by ultracentrifugation (100,000 × *g* for 20 min at 4°C) and the supernatant recovered.

Synthesis of granule proteins in the three cell populations was examined by immunoprecipitation with "anti-granule protein" antibodies coupled to CNBr-activated Sepharose 4B beads (Amersham-Phamacia Biotech) at 5 mg IgG/ml gel. Immunoprecipation was performed by adding 25 μl IgG-coupled Sepharose beads per milliliter cell lysate and incubating at 4°C for 2 h. The Sepharose beads were recovered by centrifugation and washed four times in RIPA buffer and twice in PBS. The immunoprecipitated proteins were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) by boiling the Sepharose particles in 100 μl SDS sample buffer and running the samples under reducing conditions. After fixation, the gels were stained by Coomassie blue, destained, and subjected to fluorography using Amplify™ (Amersham-Phamacia Biotech) and exposure of the dried gels to Kodak X-Omatic AR at −80°C for 1–3 days.

The biosynthesis data demonstrated that MPO and elastase, which are stored in azurophil granula, were synthesized at the myeloblast/promyelocyte stages of neutrophil differentiation (Borregaard et al., 1995; Sørensen et al., 1997). The majority of the specific granule proteins lactoferrin, NGAL, and hCAP-18 was synthesized in myelocytes/metamyelocytes ("band 2" cells) (Borregaard et al., 1995; Sørensen et al., 1997) whereas gelatinase was synthesized predominantly in "band 3" cells (band/segmented neutrophils) (Borregaard et al., 1995). These data fit the "targeting-by-timing" model for sorting of neutrophil granule proteins (Le Cabec et al., 1996; Borregaard and Cowland, 1997). This model postulates that it is the timing of protein synthesis during neutrophil maturation that determines in which type of granule the proteins are stored, i.e. proteins synthesized in promyelocytes will end up in azurophil granula whereas proteins synthesized in myelocytes will end up in specific granula. The biosynthesis patterns of MPO, NGAL, and gelatinase in band 1, 2, and 3 cells are shown in Fig. 3.

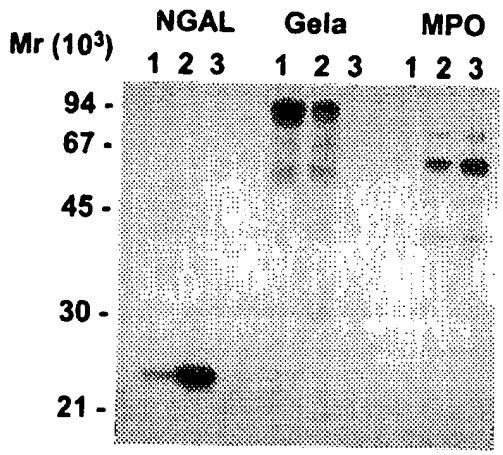

Fig. 3. Biosynthesis of granule marker proteins. Bone marrow cells from band 1, 2, and 3 were incubated with [^{35}S]-methionine for 30 min and chased for 2 h. Cell lysates were immunoprecipitated with anti-NGAL, anti-gelatinase, and anti-MPO. The immunoprecipitates were analyzed by 12% SDS–PAGE and fluorography. Reproduced from Blood with permission.

5.3. Analysis of transcripts for granule proteins in neutrophil precursors

In order to determine whether the mRNA profiles for granule proteins match the biosynthesis profiles of their cognate proteins, RNA was isolated from the three populations of neutrophil precursors for Northern blot analysis (Cowland and Borregaard, 1999). We have tried different extraction procedures and RNA purification kits and found that only RNA isolation by the acid–phenol protocol (Chomczynski and Sacchi, 1987) gave reproducible results. In short, all cells from each of the three bands were lysed in 5.5 ml GITC buffer (3.6 M guanidinium isothiocyanate, 180 mM Na-Acetate (pH 4.1), 1% (vol/vol) β-mercaptoethanol) and the solution homogenized by drawing it through a G-21 needle on a 20-ml syringe 15–20 times. The RNA was extracted twice with 6 ml phenol:chloroform:isoamylalcohol (100:49:1) and the RNA precipitated by the addition of 1 vol. isopropanol. The pellet was resuspended in 600 μl GITC buffer and precipitated with isopropanol. The pellet was washed carefully with 500 μl cold 70% ethanol and resuspended in 20 μl 0.1 mM EDTA.

For Northern blot, 10 μg of RNA was run on a 1% agarose-gel with 6% formaldehyde in 1 × MOPS (20 mM 3-(*N*-morpholino)-propane-sulfonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0). The RNA was transferred to a Hybond-N membrane (Amersham-Pharmacia Biotech) by capillary blotting and fixed by UV-irradiation. The filters were pre-hybridized for 2–3 h at 42°C in 30 ml hybridization buffer (50% deionised formamide, 1 g/l BSA, 1 g/l Ficoll 400, 1 g/l polyvinylpyrolidon, 0.5% SDS, 0.9 M NaCl, 50 mM NaH_2PO_4, and 5 mM EDTA (pH 7.5)) with 20 μg/ml denatured salmon sperm DNA. The cDNA probes used for hybridization were ^{32}P-labeled with the Random Primers DNA Labeling System (Gibco BRL) and hybridized to the membranes overnight. The membranes were washed for 2 × 15 min at 60°C in 2 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate (pH 7.0)), 0.5% SDS followed by 2 × 15 min in 0.2 × SSC, 0.5% SDS. The sizes of the mRNAs were determined by reference to 18S and 28S ribosomal RNA. The blot was developed and quantified by a phosphoimager (FUJI X Bas 2000) using the software TINA ver. 2.086 (Santax, Aarhus, Denmark).

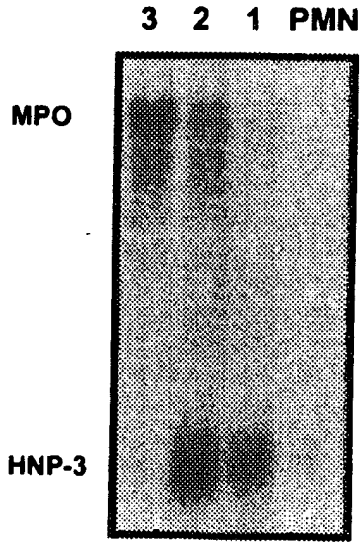

Fig. 4. Northern blot of total RNA from mature granulocytes from peripheral blood (PMN) and three populations of neutrophil precursors from bone marrow (band 1, 2, and 3). The blot was hybridized with probes against two matrix proteins of azurophil granules: MPO and defensin (HNP-3).

The mRNA distribution for 16 different granule proteins was determined in the three Percoll-separated neutrophil precursor populations and in neutrophils from peripheral blood (Cowland and Borregaard, 1999). In the cases where the biosynthetic profiles of the granule proteins were known, the mRNA profiles were found to match these perfectly indicating that the expression of granule proteins is determined by the expression of their cognate mRNAs. In the remaining cases, the mRNAs had the cellular distribution one would expect if their cognate proteins were to be directed to their known storage organelles according to the targeting-by-timing model. A representative Northern blot hybridized with cDNA probes against MPO and defensin is shown in Fig. 4.

6. Conclusion

Although different techniques for isolation of neutrophil precursors from bone marrow exist, density centrifugation on a Percoll gradient is currently the only method that has been used for separating neutrophil precursors for the study of protein biosynthesis and mRNA expression. Percoll separation is a very reproducible and robust method and has been used with success to investigate the levels of mRNA expression and the amount of neutrophil granule protein biosynthesis during myelopoiesis. For these reasons, Percoll separation of neutrophil precursors is at present the method of choice for such studies.

Acknowledgements

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